IN VITRO ACETYLATION OF HISTONES IN RAT LIVER CHROMATIN

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Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Rat liver chromatin or deoxyribonucleoprotein (DNP) was found to exhibit acetyltransferase activity *in vitro*, which seems to be closely associated with it. Evidence is provided which indicates that this transfer of acetate from acetyl-CoA to lysine in "arginine-rich" histones represents an enzymatic reaction. A number of comparisons have revealed that the *in vitro* reaction occurring within DNP is much less random than the spontaneous acetylation of free histones in solution in the presence of acetyl-CoA. Reports from other laboratories have indicated that a similar enzyme could be extracted from an acetone powder of either whole liver or nuclei. The study reported seems to involve a different enzyme since no acetyltransferase activity can be obtained from an acetone powder of chromatin using these methods. A procedure similar to that used for the extraction of mammalian RNA polymerase has been successfully applied to the extraction of acetyltransferase activity from rat liver

chromatin, although even in this case, about half of the activity remains bound to the DNP complex. The extraction of acetyltransferase activity from chromatin provides strong support for the assumption that the described *in vitro* reaction is enzymatic.

INTRODUCTION

The mechanism of gene control remains today as one of the major unexplained areas of biology. It is obvious from an analysis of living systems that some form of control must exist. One example is the circadian rhythm of liver enzyme activity exhibited by rats and other mammals.[1] Although oscillations in the activity of some enzymes may be explained by enzyme stimulation or inhibition, many enzymes are subject to induction and repression at the level of the genome. In this case the production of a new enzyme is dependent on deoxyribonucleic acid (DNA) directed ribonucleic acid (RNA) synthesis as an initial step. The mechanism by which the control of the expression of certain regions of the genome occurs in eukaryotic cells is largely unknown. In prokaryotic cells a model system has been proposed involving repressor proteins which interact with DNA, thereby influencing its transcription by RNA polymerase.[2-6] Small effector molecules interact with the repressor protein, changing its configuration in such a way as to influence its interaction with DNA and thereby the

transcriptive process. These effector molecules can be metabolites or other substances capable of allowing the cell to become responsive and adaptable to changes in its environment. It is not known whether a similar mechanism is operative in eukaryotic cells.

The differentiation process undergone by eukaryotic systems is another area where gene control is operative. Here a totipotent cell produces offspring of a particular type, the characteristics of which are maintained from one generation to another. Since within an organism each cell type contains the same amount of DNA and hybridization studies have shown the DNA of each cell type to be similar to that of every other type,[7-10] the question arises as to how the cells acquire and maintain the selective expression of specific genetic information from one generation to another. The eukaryotic system is therefore more complicated than the prokaryotic cell in that it is not only capable of reversible changes in gene expression, such as occurs in enzyme induction, but also undergoes more permanent changes which are relatively constant from generation to generation. That these changes are not completely irreversible has been shown by nuclear transplantation studies wherein nuclei from the intestinal cells of Xenopus appeared to be capable of supporting the development of a complete

embryo from an enucleated egg.[11] However, Briggs and King have demonstrated that nuclei taken from frog embryo cells at successive stages of development are progressively less able to support normal development.[12] Although the total complement of genetic information is present, the expression of this information is restricted by its passage through developing cytoplasm.

Composition of the Interphase Nucleus

Attempts to explain the mechanism of genetic control must take into consideration the structure of the nucleus. In the eukaryotic cell the nucleus is a defined area surrounded by a semipermeable membrane. This membrane influences a dynamic interchange of materials between the nucleus and the cytoplasm. Within the nucleus is contained DNA and its associated proteins. Nuclear sap proteins can be removed by isotonic saline washes whereas other proteins along with the DNA form part of the insoluble chromatin complex.[13] In the interphase nucleus part of the chromatin has a diffuse appearance and consists of many fibers. In some regions the chromatin appears in a more condensed condition. Condensed regions of a single chromosome are called heterochromatin, as opposed to the less condensed or euchromatic regions. The term "heterochromatin" is also used to refer to a condensed state of chromatin, and is applied to such regions of the interphase nucleus as well.[14]

The components of the chromatin include DNA and histone proteins, found in approximately equivalent amounts.[15, 16] Nonhistone proteins comprise about 27 percent of the DNA associated protein in calf thymus,[17] and 43 percent in liver.[18] These proteins are subdivided into acidic proteins, which are alkali soluble, and residual proteins, which remain behind after acidic extraction of histones and basic extraction of acidic proteins.[19] RNA polymerase and other enzymes are among those which make up the nonhistone chromosomal proteins.[20, 21] Also some species of RNA have been found associated with the chromatin.[22, 23]

The arrangement of the chromosome has been a subject of considerable controversy among cytologists. Interphase chromatin fibers of 200-300 A^{o} in diameter have been described and Ris has proposed that these consist of two 100 A^{o} units, and that each of these further consist of two 40 A^{o} units representing single DNA-protein molecules.[24, 25] This has been questioned and more recent data utilizing the techniques of trypsin digestion of chromatin fibers in conjunction with electron microscopy shows that each 230 A^{o} fiber consists of a single DNA molecule packed in a proteinaceous sheath.[26, 27] According to the model this unit is further induced to supercoil by the addition of more protein.[28] Different chromosomal proteins may play specific roles in the maintenance of this structure.

Restriction of the Genome

The need for selective gene expression implies that certain regions of the chromsome will be active at a given time whereas others will remain quiescent. Evidence that this is the case comes from hybridization studies. Georgiev observed that RNA synthesized on free DNA hybridized to a much greater extent with DNA than that synthesized on chromatin.[29] He explained this by the observation that there are many repeated DNA sequences in the chromatin which are restricted in transcription. However, RNA synthesized on chromatin competes with *in vivo* synthesized messenger RNA (mRNA) for sites on DNA more efficiently than does RNA synthesized on free DNA. This implies that RNA synthesized on a chromatin template more closely approximates the *in vivo* situation.

Heterochromatic or tightly condensed regions of chromatin are considered to be in the repressed condition, whereas the more diffuse euchromatic regions are believed to be metabolically active. Electron microscope autoradiography has revealed that the diffuse, presumable euchromatic, regions actively incorporate ^{3}H-uridine in contrast to the heterochromatic regions.[30] Frenster has isolated bulk fractions of condensed and diffuse chromatin from interphase calf thymus lymphocytes.[31] He has shown that the condensed

regions contain up to 80 percent of the nuclear DNA, but only 14 percent of the nuclear RNA. This observation would seem to support the concept that the condensed state of the chromatin is associated with repression of genetic expression.

Possible Mechanisms for Gene Control

Repetition of DNA sequences

One finding which has recently attracted attention is that more than one third of the DNA of higher organisms is made up of sequences which recur anywhere from a 1×10^3 - 1×10^6 times per cell.[32] The nucleolus of amphibian oocytes contains many replicas of the genes which code for ribosomal RNA.[33, 34] This repetition of specific genes has been viewed as a unique means of controlling the expression of genetic information by allowing for the production of certain products in greater quantity at a given time in the life of the cell.

RNA polymerase and associated factors

In considering the ways in which the regulation of gene expression may occur, RNA polymerase and its associated sigma factor must be mentioned. In bacterial systems the sigma factor has been found to be responsible for the initiation of RNA chains by DNA-dependent RNA polymerase.[35] RNA synthesis in isolated nuclei of avian erthryocytes was stimulated by a heat stable factor present in extracts of

HeLa cells.[36] More recently, a protein-like factor from calf thymus with a sedimentation constant of 3 S has been described.[37] This factor stimulated DNA-dependent RNA synthesis from the same tissue after the enzyme had already become bound to DNA. These findings have a bearing on explanations of gene control in that specificity of initiation site and rate of RNA synthesis are influenced by these factors.

Histones as repressors

In 1951 the Stedmans suggested that histones may play a role in the regulation of gene activity by acting as repressors of RNA synthesis.[38] The observation by Huang and Bonner that histones suppress RNA polymerase activity when added to DNA *in vitro* prompted an increased investigation into this area.[39] The relevancy of these findings has been questioned and it has been suggested that the inhibition of RNA polymerase activity is due simply to the precipitation and removal of DNA from solution.[40] Bonner and Huang have answered this objection by describing conditions which prevent DNA precipitation,[41] and Butler and Chipperfield showed that the amount of inhibition continues to increase with increasing histone concentrations even after the DNA has been fully precipitated.[42] Clark and Byvoet attempted to resolve the question of whether or not a corre-

lation exists between the activity and the solubility of the DNA template by plotting the logarithm of the percent inhibition of template activity against the amount of unaggregated DNA present in the reaction mixtures at various histone/DNA ratios.[43] This type of plot revealed a close correlation between the solubility of the DNA template and the inhibition of polymerase activity by histone. In any case, it seems likely that reconstitution of the DNA-histone complex by simple titration of DNA with histones preserves very little of the specificity of the original relationship and there is a question as to whether or not these *in vitro* findings have any relation to the *in vivo* situation.

Further support for the involvement of histones in repression of RNA synthesis came from studies which selectively removed different histone fractions from chromatin. Successive extractions of pea-bud chromatin with NaCl solutions resulted in increased template activity.[44] Extraction of histones from the heterochromatin of the male mealy bug was shown to derepress RNA synthesis and to increase actinomycin D binding.[45] When all the histone was removed from the heterochromatin it became equivalent to the euchromatin in these respects.

Nonhistone chromosomal proteins as antagonists to DNA-histone interaction

It has been suggested that nonhistone proteins act as antagonists to the DNA-histone interaction which serves to repress RNA synthesis.[46] In support of this, it has been found that basic proteins, such as histones, are closely associated with DNA, occurring in highest concentrations where DNA appears to be tightly coiled and condensed.[47] During puff formation in dipteran giant salivary chromosomes, i.e., at regions of intense RNA synthesis, there is no measurable difference in the amount of stainable basic protein, implying a continuity of structure during the transcriptive process. Interestingly, however, there seems to be an increase in the acidic proteins in these regions during puff formation.[48] In isolated chromatin fractions the ratio of total histones to DNA did not significantly differ within either the repressed or active fractions, and the relative proportions of each of the distinct types of histones were similar in both forms of chromatin. However, when nuclear polyanion contents of active and repressed chromatin fractions were determined relative to the DNA contents, the active fractions were found to contain more total nonhistone proteins, RNA and phospholipids, and phosphoprotein phosphorous.[49-53] About 15 percent of the nonhistone proteins consist of such nuclear phosphoproteins.[54-55]

In vitro experiments have shown that acidic proteins complex with free histones and prevent histone inhibition of RNA synthesis by bacterial RNA polymerase.[56] However, these proteins cannot dissociate the histone-DNA complex to reverse histone inhibition of RNA synthesis. Therefore, Spelsberg and Hnilica have suggested that their action may reside in the prevention of DNA-histone interaction rather than the dissociation of DNA-histone complexes.[56]

It has been shown by Paul and Gilmour that all the protein components of the chromosome are necessary for the production of RNA which resembles that found *in vivo*.[57] Using hybridization techniques they found that histone repression of DNA directed RNA synthesis *in vitro* is nonspecific. However, if they added the residual and acidic proteins back to DNA and histone, the new mRNA synthesized does compete effectively with mRNA produced *in vivo*. Similar experiments by Bekhor, Kung, and Bonner showed that DNA, histones and chromosomal RNA produced a primer capable of yielding mRNA equivalent to the natural product.[58] They therefore suggested that nonhistone protein is not repressive. Marushige, Brutlag, and Bonner, in contrast to Paul and Gilmour, found that DNA saturated with nonhistone chromosomal proteins was as good a primer as naked DNA.[59] These differences may result from contaminated preparations

and have yet to be resolved. It is difficult to imagine a mechanism for gene control in which any one of the elements of the chromosome does not play a role, either directly or indirectly. The observations of these interactions is, therefore, limited probably by experimental technique.

Nonhistone chromosomal proteins as antagonists to RNA polymerase-histone interaction

Association of chromosomal proteins with RNA polymerase is another means by which repression of transcription could take place. Histones form complexes with bacterial and mammalian RNA polymerase leading to the inhibition of *in vitro* RNA synthesis.[60-62] Since it has been observed that salt and polyanions (e.g., acidic nuclear proteins) activate RNA polymerase in chromatin,[63, 64] it has been suggested that this may be due to dissociation of enzyme-histone complexes, thereby stimulating RNA synthesis.[65] This suggestion is supported by the observation that the ionic strength required for maximum dissociation of the enzyme-histone complex is within the range required for the activation of endogenous RNA polymerase. In contrast, no detectable dissociation of DNA-histone complexes was seen at these ionic strengths.

Arguments against histones as repressors

One of the strongest criticisms against the idea that histones function as genetic repressors is the fact that

histones lack specificity. There are five main histone fractions: very lysine-rich (f_1), moderately lysine-rich (f_{2b}), and the arginine-rich (f_{2a1}, f_{2a2}, and f_3) histones.* Although there are major differences between the five histone fractions,[66-69] there is very little difference in the composition of similar fractions from different species. Sequence studies of an arginine-fraction (f_{2a1}) from organisms as far removed from each other as calf (thymus) and pea seeds revealed essentially identical primary structures.[70-75] Recent data on very lysine-rich (f_1) histones showed that they are more numerous and have some phenotypic specificity,[76-79] but not enough to account for the observed phenotypic differences. It has been suggested, therefore, that chromosomal RNA plays a role in gene repression and derepression.[46] This is an attractive idea since it offers a means for specific recognition of DNA sites for selected repression of transcription. In support of this, Huang and

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*Older terminology referred to just the "arginine-rich" histones, which included the moderately lysine-rich (f_{2b}) fraction, and the "lysine-rich" histones, which only referred to the very lysine-rich (f_1) fraction. Hereafter, "arginine-rich" enclosed in quotation marks refers to the arginine-rich (f_{2a1}, f_{2a2}, and f_3) and moderately lysine-rich (f_{2b}) fractions combined.

Bonner have found a chromosomal RNA bound to a nonhistone protein,[22, 23] which they have postulated is associated in complexes with histones.[80]

Another major objection to the simple repression of transcription by histone binding to DNA comes from the observations that histones turn over at the same rate as DNA,[81-83] are synthesized at about the same time of the cell cycle as DNA,[84-88] and are present at the same levels within the chromosome during RNA synthesis.[31] These observations mediate against a simple explanation of histones acting alone as gene repressors simply by combination with and dissociation from DNA. It is for these reasons that attention has focused on structural modifications of histones, taking place after their synthesis, which may influence their binding to DNA, and thereby perhaps the transcriptive process.[89]

Modification of histone structure

Three methods of histone modification are known. These are methylation of lysine and histidine residues,[90-92] acetylation of lysine residues,[71, 74, 93, 94] and phosphorylation of serine residues.[95]

Methylation of Histones

Isolated nuclei from calf thymus and chromatin from peas,[96-98] rat liver,[90] and Ehrlich ascites cells can

methylate histones *in vitro*.[91] Methionine is the methyl donor via S-adenosyl-methionine.[97] Gershey *et al.* have found that the methylation of histones occurs much more rapidly in immature erythroid cells, but as the nucleus matures and loses its biosynthetic capacities, it also loses its capacity to methylate histidine in its histones.[92] Tidwell *et al.* have compared the time courses of histone synthesis and methylation during regeneration of the liver in the rat.[99] They found that maximal histone methylation occurs at a time when the rates of histone and DNA synthesis have already begun to decline and does not correlate with an increase in RNA or nonhistone protein synthesis. They suggest, therefore, that methylation is not involved in gene activation for RNA synthesis.

Phosphorylation of Histones

The phosphorylation of histones occurs both *in vivo* and *in vitro*. Ord and Stocken have found a 2-fold increase in the rate of phosphorylation and phosphate content of very lysine-rich (f_1) histone of regenerating rat liver near the first peak of DNA synthesis after partial hepatectomy.[100] Langan and Smith have isolated an enzyme from liver which specifically transfers phosphate from adenosine triphosphate (ATP) to serine residues of protamines and histones.[95] The most rapidly phosphorylated histones are the lysine-rich

fractions, f_1 and f_{2b}. It appears that there is more than one histone kinase and that they differ in their rate of phosphorylation of f_1 and f_{2b} histones.[101] A phosphatase has also been partially purified which is specific for phosphorylated histones and protamine.[102]

Since phosphorylation by histone kinase is stimulated by adenosine-3', 5'-monophosphate (cyclic AMP) Langan has suggested a mechanism for the induction of RNA synthesis by those hormones that cause increases in the concentration of cyclic AMP.[103] The administration of glucagon or insulin to rats causes an *in vivo* increase in phosphorylation of lysine-rich histones in the liver involving the same specific serine residue that becomes phosphorylated *in vitro*.[104]

The phosphorylation of chromosomal proteins is not restricted to histones. Kleinsmith *et al.* have observed an increase in ^{32}P-phosphate incorporation into nuclear phosphoproteins of lymphocytes stimulated to grow and divide by phytohemagglutinin.[105] This increase was particularly evident in nonhistone protein and occurs prior to an increase in the rate of RNA synthesis occuring under these conditions. Gershey and Kleinsmith have followed changes in nuclear phosphoproteins during the course of development of the avian erythrocyte.[106] As maturation proceeds, the nuclear levels of both phosphoprotein kinase and protein-

bound phosphorous correlate with a decrease in the biosynthetic activities of the nucleus. The bulk of the incorporation of phosphate occurs as in the case of the lymphocyte in the nonhistone protein fraction. Since Langan has reported that phosphoproteins can interact with histones *in vitro*, and that complex formation with phosphoprotein decreased the inhibitory effects of added histone on DNA dependent RNA synthesis, it has been suggested that this interaction may play a role in the regulation of gene expression.[101] Further support for this idea comes from studies on isolated chromatin fractions, which revealed that phosphoprotein concentrations are highest in fractions which are active in RNA synthesis.[107] Dipteran salivary gland chromosomes also show active phosphorylation of nonhistone proteins, particularly in the puff regions.[108]

Acetylation of Histones

The acetylation of histones has been studied in intact animals,[109, 110] in cells in tissue culture,[111-113] in isolated nuclei,[94, 96, 114-116] and in isolated enzyme preparations.[117-119] Early studies measured the incorporation of ^{14}C-acetate, but it was later found that acetyl Coenzyme A (acetyl-CoA) is the direct acetyl donor.[114] In every case examined the incorporation of labeled acetate occurred primarily in the arginine-rich (f_3, f_{2a1}, and f_{2a2}) fractions,

while very lysine-rich (f_1) and moderately lysine-rich (f_{2b}) fractions incorporated minor amounts. This is in contrast to the predominant phosphorylation of the lysine-rich fractions.[101] The low level of acetate incorporation into thymus f_1 histone also contrasts with the finding that this fraction is almost completely acetylated at its amino terminal end.[120, 121] These alpha-N-acetyl groups appear to be relatively stable, whereas the internal epsilon-N-acetyl groups in the f_{2a1} and f_3 fractions are involved in a dynamic process of acetylation and deacetylation. Evidence for the fact that acetyl groups are attached to histones after synthesis of the polypeptide chain is provided by the finding that puromycin in concentrations sufficient to block protein synthesis in calf thymus nuclei did not inhibit the acetylation of histones.[96] In agreement with this, Byvoet has observed that the turnover of acetyl groups in histones is much more rapid than the turnover of histones themselves and the ratio of the two turnover rates vary with the tissues.[81] Gershey et al. have prepared histone fractions from calf thymus nuclei which had been incubated in the presence of sodium-^{14}C-acetate.[93] These were digested with trypsin and pronase and the resulting peptides and amino acids were separated by chromatography. Only the arginine-rich fractions f_{2a1} and f_3 were appreciably labeled and the radioactivity was associated with

epsilon-N-acetyl-lysine. Studies on the sequence of f_{2a1} histones have shown that 50 percent of the lysine residues at position 16 are acetylated in calf thymus,[71] whereas in pea bud the epsilon-N-acetyl content is only 6 percent.[74]

Enzyme fractions which acetylate histones have been isolated from an acetone powder of whole pigeon liver.[117] Two fractions were found, of which one activates acetate by converting it in the presence of ATP, Mg^{++}, and coenzyme A to acetyl-CoA, while the other transfers the acetate from acetyl-CoA to histones. Using similar procedures Gallwitz reported the isolation of acetokinase from an acetone powder of rat liver nuclei.[118] This enzyme was capable of transferring acetate from acetyl-CoA to isolated histones and was especially active for arginine-rich (f_3 and f_{2a1}) fractions. More recently Gallwitz reported the isolation of two histone-specific transacetylases from rat liver nuclei using a different approach.[119] Rat liver nuclei were sonicated in the presence of 1 M ammonium sulfate and 20 percent glycerol, followed by precipitation with 3.5 M ammonium sulfate. A 50-fold purification was accomplished by chromatography. No preference of the two enzymes has been found for one specific histone fraction.

The isolation of a deacetylating enzyme was reported by Inoue and Fujimoto from a salt extract of calf thymus.[122]

This enzyme showed a preference for histones which were acetylated by incubating calf thymus nuclei with ^{14}C-acetic anhydride. Libby has also reported deacetylating activity in nuclei of rat liver and Novikoff hepatoma.[123]

Studies by Allfrey, Mirsky, and their colleagues have attempted to correlate histone acetylation and chromosomal activity. Arginine-rich histones from calf thymus inhibited RNA polymerase in an "aggregate enzyme" from calf thymus as well as purified RNA polymerase from bacterial sources.[124, 125] In the case of the mammalian RNA polymerase studies, the problem was complicated by difficulties in the purification of the enzyme because it remained bound to the chromatin complex.[124] When external histones are added to this complex, inhibition of RNA synthesis is observed, but the significance of this observation is questionable and it may represent a nonspecific effect. When RNA polymerase from Escherichia coli was used, this problem was circumvented as it can be obtained in purified form. In vitro RNA synthesis by E. Coli RNA polymerase was followed using ^{14}C-ATP (adenosine triphosphate) or ^{14}C-UTP (uridine triphosphate) as precursors. It was found that the incorporation into RNA was decreased by the addition of arginine-rich histone. This degree of inhibition of RNA synthesis was progressively decreased experimentally by the chemical addition of acetyl

groups to the isolated histones.[96] The technique used to acetylate the histones for the inhibition of RNA snythesis was selected to give maximum acetylation of alpha amino groups and minimum acetylation of epsilon amino groups of lysine. It has subsequently been found that *in vivo* acetylation involves primarily the epsilon amino group of lysine. Therefore, it would be interesting to study this effect using histones which have been acetylated in a more physiological manner.

Histone acetylation has been compared in chromatin fractions which are active or inactive in RNA synthesis. Fractionation of thymus chromatin after labeling nuclei *in vitro* with isotopic RNA precursors has shown that RNA synthesis proceeds faster in the diffuse chromatin than in the condensed chromatin.[30, 31] Comparisons of the rates of acetate incorporation into the histones of each of these fractions show that histone acetylation, like that of RNA synthesis, proceeded 2-3 times more rapidly in the diffuse chromatin fraction. Autoradiography of calf thymus nuclei after ^{3}H-acetate incorporation indicates that acetylation occurs in both diffuse and condensed regions but is particularly evident at the boundaries between the two regions.[126] This would be expected if acetylation is associated with the transition of chromatin from a condensed to a more diffuse, active state.

Since puffing in dipteran polytene chromosomes has been associated with RNA synthesis, attempts to correlate this with acetylation of histones have been made.[127] Autoradiographic studies indicated uptake of radioactivity in chromosomes when salivary glands of Chironomus thummi were incubated in the presence of ^{3}H-acetate. The grain densities over the puff regions however were not particularly intense and Allfrey has suggested that the methods used in the fixation process extract most of the acetylated arginine-rich histones from the chromatin. However, using new methods of fixation, which retain incorporated acetate, Clever and Ellgaard also failed to observe any accumulation of label over puff regions and it is their opinion that puff formation does not include acetylation of histones.[128]

The mature nucleated red cells of birds synthesize little or no RNA, whereas lymphocytes do. Comparisons have been made of the rates of histone acetylation and RNA synthesis in nucleated erythrocytes and in isolated lymphocyte nuclei. These reveal that the lymphocyte nuclei were far more active in acetylating their histones than were the erythrocytes.[126] Although this may be coincidental, it is further evidence for the hypothesis that acetylation of histones may be associated with gene activation.

Comparison of the rates of histone acetylation and RNA

synthesis in lymphocytes at different times after the addition of phytohemagglutinin (PHA) to the culture medium shows that RNA synthesis is stimulated and that there is an increase in acetylation of arginine-rich histones preceding the increase in RNA synthetic activity.[111] In contrast to the behavior of lymphocytes upon exposure to PHA, polymorphonuclear leucocytes curtail the synthesis of RNA and under these conditions histone acetylation is also depressed.[111, 112] Monjardino and MacGillivray have suggested, however, that these effects may be nonspecific since some preparations of PHA were found to increase RNA synthesis in lymphocytes while decreasing histone acetylation.[129] Killander and Rigler have shown that the amount of acridine orange dye bound to the chromatin of PHA stimulated lymphocytes increased rapidly over a time course which is similar to that for histone acetylation.[130] This reflects a change in chromatin molecular structure as a result of PHA treatment, which as suggested by Allfrey may be initiated by the acetylation reaction.[126]

It has been shown that in liver cells gene activation occurs with consequent appearance of new species of RNA as a result of partial hepatectomy.[131] Increases in DNA template activity of isolated liver nuclei,[132] and increases in template activity and RNA polymerase activity in chro-

matin and in "aggregate" enzyme preparations have been reported.[44, 133-135] With this in mind Allfrey and others have studied the acetylation of histones during the course of liver regeneration.[110, 126] The specific activity of different histone fractions from control and regenerating rat liver were measured at various time intervals after an *in vivo* injection of ^{3}H-acetate. The results in sham-operated controls indicated a high rate of acetate uptake, the maximum specific activity being reached in 15 minutes. Turnover at 60 minutes was such that only one third of the acetyl-groups originally incorporated into the arginine-rich fraction were left. This pattern was altered for regenerating liver. In this case histone acetylation was increased by 300 percent at 3 hours after partial hepatectomy and in the period between 1-2 hours after the operation the histones lost 13.7 percent of their original acetyl content while the controls had lost 70 percent. Allfrey has suggested that this is due to an increase in the rate of acetylation and lower rates of deacetylation for regenerating liver. The increase in the percent retention of the acetyl groups in histones from regenerating liver just precedes a rise in RNA polymerase activity in regenerating rat liver nuclei and reaches its peak 2 hours before the nuclei reach the first plateau in RNA polymerase activity. These findings

are consistent with the view that acetylation of histones modifies DNA-histone interactions, and the subsequent changes influence the template activity of the chromatin for RNA synthesis.

A correlation has also been observed between the patterns of RNA synthesis and histone acetylation in liver responding to stimulation by steroid hormones.[109, 126] Administration of cortisol to adrenalectomized rats leads to increases in the amounts and changes in the types of RNA synthesized. As in the case of liver regeneration, cortisol treatment of adrenalectomized rats leads to early increases in the rate of acetylation of arginine-rich histones and a suppression of turnover of previously incorporated acetyl groups. Cortisol stimulation of the liver is only one example of hormone-induced gene activation. Takaku et al. observed increases in the acetylation of histones in spleen cells of polycythemic mice just preceding an increase in RNA synthesis at 4 and 8 hours after erythropoietin injection.[136] Another example is provided by estradiol which increases RNA synthesis in the uterus.[137-139] Estradiol has also been reported to stimulate the acetylation of histones by cell free extracts of the rat uterus.[140]

Rationale of Proposed Study

Although none of these observations show a direct cause

and effect relationship, they suggest that the acetylation of histones may be related to genetic expression. The fact that only two of the five known histone fractions are acetylated upon incubation of nuclei in the presence of radioactive acetate suggests that this is an extremely specific reaction. The complete elucidation of the amino acid sequence of the f_{2a1} histone has even established that in the entire molecule only one specific lysine residue is acetylated at the epsilon amino position.[71, 74] It does not seem likely that the acetylation of only one lysine residue would change the structure of the histone sufficiently to influence gene activity. However, the location of this lysine residue within an unusual cluster of five basic residues would seem rather suggestive of some role which this acetylation may play in histone-DNA interaction.

It appeared that the biological implications of the postulated changes in histone-DNA interactions, resulting from histone acetylation, are sufficiently important to warrant a thorough study of this process at the molecular level. In order to carry out such a study, the development of an *in vitro* system is essential in view of the many advantages of such a system over the myriad of possible indirect effects which may influence the outcome of *in vivo* studies. Some of these may be hormonal influences, pool

sizes, and degradation of labeled precursor. Even in the case of isolated nuclei there is a possibility of cytoplasmic contamination. In addition, nuclei contain an enzyme which deacetylates histones (see Addendum). The development of the *in vitro* system described in this study which utilizes deoxyribonucleoprotein (DNP) has eliminated many of these disadvantages and possesses all the advantages of *in vitro* systems in general. The most important of these is the isolation of the enzyme to be studied from the many variables which cannot be controlled *in vivo*. Other advantages are greater reproducibility, control of medium composition, temperature, and pH. In the first stages of the development of this system, it appeared that DNP could be employed instead of isolated nuclei, especially if the most immediate precursor, acetyl-CoA was used. It was found that isolated rat liver DNP was capable of transferring acetate from acetyl-CoA to histones. A problem which presented itself at this point was the possibility that this transfer might not be enzymatically catalyzed since it was found that free histones are spontaneously acetylated upon incubation with acetyl-CoA. It, therefore, became essential to establish that this transfer is an enzymatically catalyzed reaction and attention has been directed toward this problem. This dissertation describes the conditions influencing the trans-

fer of acetate from acetyl-CoA to histones in rat liver chromatin and provides evidence that this is an enzymatic reaction.

MATERIALS AND METHODS

Isolation of Nuclei

Although in the beginning these studies were done with calf thymus, this was replaced by rat liver since fresh preparations were found to exhibit higher acetylating activities in contrast to frozen material. Other factors in favor of rat liver include availability of previously described methods for the isolation and purification of nuclei and deoxyribonucleoprotein (DNP). It was also desirable to compare experiments on Novikoff hepatoma and the tissue from which the tumor presumably originated.

Male Holtzman rats weighing approximately 300-500 gm were decapitated and the liver excised. All preparations were carried out at 4°C. The liver was homogenized in 10 volumes (weight/volume) 0.25 M sucrose containing 1.5 mM $CaCl_2$ in a Potter type homogenizer, filtered through cheese-cloth to remove connective tissue, and centrifuged at 600 x g for 10 minutes.[141] The pellet was resuspended in 5 volumes of 0.25 M sucrose, 1.5 mM $CaCl_2$ and recentifuged as above. The nuclear fraction thus obtained was purified

by resuspension and centrifugation at 40,000 x g for 30 minutes in 2.1 M sucrose, 0.5 mM $CaCl_2$.[142]

It has been found that this procedure removes surface cytoplasm from rat liver nuclei even when loose homogenizers were employed.[21] Examination of the 2.1 M sucrose, 0.5 mM $CaCl_2$ pellet by phase microscopy showed clean nuclei with little visible debris in the preparation. Nuclei have also been prepared according to more recent procedures using detergent, Triton N-101, which has been shown by electron microscopy to remove the outer nuclear membrane and perinuclear ribosomes.[143]

Where calf thymus was used, the 2.1 M sucrose purification of nuclei was omitted because there is less of a problem of cytoplasmic contamination with this tissue. If Novikoff hepatoma was used, fibrous connective tissue was removed by filtration through a wire screen rather than cheesecloth.

Isolation of Deoxyribonucleoprotein (DNP)

DNP was prepared from nuclei by homogenization in 10-15 volumes of 0.14 M NaCl, 0.01 M sodium citrate in a micro Waring Blendor (Virtis), for 1 minute followed by centrifugation at 2000 x g for 10 minutes. The sediment was resuspended by light homogenization in a loose fitting Potter type homogenizer and recentrifuged. This procedure, which

removes soluble nuclear proteins,[13] was repeated once. Chart 1 illustrates the outline of the general procedure used.

Conditions of Incubation

Unless otherwise stated, samples of isolated DNP or purified nuclei containing about 0.3-0.5 mg of histones were incubated at 37°C in a medium originally designed for studies of protein synthesis by isolated calf thymus nuclei.[144] This medium contained 0.19 M sucrose, 20 mM glucose, 25 mM phosphate buffer, pH 6.75, 12 mM NaCl, 0.75 mM Ca^{++}, 5 mM Mg^{++} and 0.01 μc acetyl-^{14}C-CoA (spec. act. 56 mc/mM, New England Nuclear), in a final volume of 2 ml (step 3 in Chart 1). In preliminary experiments ^{3}H-acetate was used as a precursor, but replaced by acetyl-^{14}C-CoA since it was found that this improved incorporation of ^{14}C-acetate. Low incorporation of acetate into histones when ^{3}H-acetate was used as a precursor may be due to lack of activating enzymes converting acetate to acetyl-CoA (see Introduction), especially since there was no stimulation of ^{3}H-acetate incorporation into histones upon the addition of ATP. Studies reported below revealed that divalent cations, glucose, and sucrose were not necessary for the transfer of acetate from acetyl-CoA to histones. They were therefore eliminated from the medium. Later, experiments were also run at a pH of 8, rather than 6.75.

After a 15 minute incubation, the reaction was stopped by cooling the flasks and adding trichloroacetic acid (TCA) to 5%. The resulting mixture was centrifuged at 2000 x g for 10 minutes (step 4 in Chart 1).

If nuclei were incubated in the above described manner, the reaction was stopped after 10 minutes by rapid cooling and centrifugation of the samples at 2000 x g for 10 minutes. The nuclei were then washed in saline-citrate to remove soluble nucleoproteins as described for the preparation of DNP.

In experiments where proteinaceous extracts were added to DNP preparations, the reaction was not stopped by TCA, but only by centrifugation after cooling, to prevent co-precipitation of the added proteins with the DNP upon addition of TCA. These proteins could contaminate subsequently extracted histones.

Extraction of Histones and Determination of Specific Radioactivity

After incubation, the total histones were extracted from the DNP sediment with 0.25 N HCl (step 5 of Chart 1). Chromatographic analysis and subsequent amino acid analysis of histones extracted in this manner have shown that less than 0.5% of the total nitrogen is due to contamination from other proteins.[145, 146] After centrifugation the supernatant was made 5% with TCA which precipitates the

"arginine-rich" (includes f_{2b} fraction) histones leaving the very lysine-rich histones behind in solution. After standing overnight the TCA precipitate was washed with acetone-1% HCl and ether and allowed to dry (step 6 in Chart 1). This procedure, therefore, selectively isolates the "arginine-rich" histones, which have been found to be by far the most actively acetylated fraction (see Introduction).

Determinations of total counts contained in histones, DNA, acidic and residual proteins (described in next section) show that 76% of the radioactive label is contained in the "arginine-rich" histone fraction and therefore cannot be ascribed to contamination with a hypothetical nonhistone fraction which is very highly labeled. Almost all of the remaining label was contained in the acidic protein fraction. Purity of histone fractions was tested by amino acid analysis and acrylamide gel electrophoresis of "arginine-rich" histones isolated according to this procedure.

In experiments where the spontaneous acetylation of free histones was studied, "arginine-rich" calf thymus histones were used. Essentially, these histones are comparable to those obtainable from rat liver,[146] but were used in place of rat liver histones because larger quantities could be obtained more easily from calf thymus. These histones were extracted from 0.14 M NaCl washed calf

thymus with 0.25 N HCl and the "arginine-rich" fractions were precipitated by 5% TCA and dried in acetone-1% HCl and ether.

Protein concentrations were determined by the method of Lowry et al. using bovine serum albumin as a standard.[147] Radioactivity was determined by liquid scintillation counting after dissolving the histones in a small aliquot of water (step 7 in Chart 1 lists this procedure). One liter of liquid scintillation fluid contained 72 ml of spectrafluor Butyl-PBD (Nuclear Chicago), 300 ml of ethanol, and 628 ml of toluene.

Determination of Acidic and Residual Proteins

Amounts of DNA in DNP subjected to various treatments were determined prior to incubation to ensure that histone/DNA ratios were unchanged by these treatments. Therefore, any differences in acetylating activity observed between pretreated DNP and control preparations cannot have been due to changes in the essential composition of the complex, such as might occur by the selective removal of histones. The precipitate remaining after the 0.25 N HCl extraction of histones was washed with cold 5% TCA, to remove any small organic molecules, such as sucrose, which might interfere with the DNA determinations. After centrifugation, the precipitate was washed with 95% ethanol, absolute ethanol,

chloroform:menthanol 2:1, and twice with ether to remove lipids. When DNA was determined by the H_2SO_4 reaction, the mixture was dissolved in 1 N NaOH and incubated at 37°C for 16-20 hours.[148] Upon acidification with HCl at 0°C the DNA precipitates leaving hydrolyzed RNA in solution. After hydrolysis in perchloric acid at 90°C for 20 minutes it was reacted with H_2SO_4 to give a colorimetric reaction.[149] The Burton test which is a modified diphenylamine test for DNA was also used.[150] In this case it was not necessary to remove RNA as this reaction is specific for deoxyribose. Specific activity of acidic proteins was determined by dissolving the proteins remaining in the precipitate after hot acid extraction in 1 N NaOH. Any residual protein undissolved by this procedure was dissolved in NCS solubilizer (Nuclear Chicago) and counted.

CHART 1: OUTLINE OF GENERAL PROCEDURE

1. Isolated nuclei washed 3x in saline-citrate→

2. Deoxyribonucleoprotein (DNP).

3. Nuclei or DNP incubated in 2 ml final volume, containing:

   0.19 M sucrose, 20 mM glucose, 25 mM phosphate buffer, pH 6.75, 12 mM NaCl, 0.75 mM Ca^{++}, 5 mM Mg^{++}, 0.01 μc acetyl-^{14}C-CoA (0.6 mμM).

4. After 15 minutes brought to 0°C and trichloroacetic acid (TCA) added to 5% and centrifuged.

5. Sediment extracted with 0.25 N HCl "Arginine-rich histones".

6. Precipitated with 5% TCA, washed with acetone-1% HCl, ether, dry.

7. Dissolved in 0.6 ml H_2O, protein concentration (Lowry), radioactivity (liquid scintillation), DNA (H_2SO_4).

Methods for Extraction of Acetylating Enzymes

Extraction of acetylating enzyme from acetone powder of rat liver nuclei

Nohara *et al.* have reported the isolation of acetylating enzymes from an acetone powder of whole pigeon liver.[117] In addition Gallwitz has extracted an acetokinase from an acetone powder of rat liver nuclei.[118] An attempt was therefore made to use a similar procedure to extract the acetyltransferase from nuclei and DNP. A modification of the original procedure was used, which was claimed by Bondy and Roberts to be successful in the isolation of a histone acetokinase from rat brain and liver nuclei.[151]

Nuclei or DNP obtained from 25 gm of rat liver were suspended in 7 ml of water. This preparation was then added dropwise with continuous stirring to 70 ml of acetone kept at -30°C. The resulting suspension was passed through Whatman No. 1 filter paper on a Buchner funnel. The precipitate was washed 3 times with 50 ml acetone at -30°C and dried in a dessicator at 0°C. The dry powder obtained was then homogenized in 13 ml of 0.1 M tris-HCl buffer at pH 8.2 and centrifuged at 2000 x g for 10 minutes. The resulting supernatant was treated with neutral saturated ammonium sulfate. The fraction which precipitated between 35-60% saturation with ammonium sulfate was dissolved in 6.5 ml of tris buffer and dialyzed against two successive 500 ml

portions of a solution which contained 0.068 M KCl, 0.001 M 2-mercaptoethanol and 0.02 M $NaHCO_3$ at pH 8.0 for 1 hour each. This dialysed preparation contained the soluble acetylating enzyme and 1 ml containing 0.1-0.5 mg of protein was incubated at 37°C with 0.5 mg of isolated "arginine-rich" calf thymus histones in 25 mM phosphate buffer, pH 8, 12 mM NaCl in the presence of acetyl-^{14}C-CoA. The reaction was stopped by precipitating the protein onto filter paper with cold 15% TCA.

In a preliminary experiment Bio Gel filters were used, which were washed twice with 2 ml of incubation medium containing 10 times the original concentration of unlabeled acetyl-CoA, twice with 2 ml of acetone-1% HCl, and twice with 1 ml of ether. Since this filter paper did not dissolve in scintillation fluid, corrections were made for loss of counts by counting a sample before and after absorption onto filter paper. In subsequent experiments millipore (AAWP02500) filters were used which could be dissolved in Bray's scintillation fluid.[152] These were dried at 90°C for 15 minutes after the TCA precipitation step and subsequently counted.

Extraction of acetylating enzyme from rat liver nuclei with saline

Johns and Forrester have found that acidic proteins

which remain bound to the chromatin complex during the 0.14 M NaCl procedures used to prepare DNP from nuclei can be removed by extraction with 0.35 M NaCl.[153] It seemed plausible that the acetylating enzyme was among this group of proteins and could be extracted from the DNP complex with 0.35 M NaCl. Therefore, attempts were made to extract the acetyltransferase with 0.35 M NaCl from nuclei and DNP.

Purified rat liver nuclei or DNP from 6 gm of liver were therefore blended for 1 minute in the Virtis homogenizer in 4 ml of 0.35 M NaCl. After a preliminary centrifugation at 2000 x g for 10 minutes to remove the major portion of the DNP, the supernatant was diluted to 0.14 M NaCl and centrifuged at 40,000 x g for 30 minutes. To check whether any DNP remained in the supernatant, the DNA concentration of the supernatants was determined which showed less than 0.15 mg of total DNA present. This supernatant was then used as an enzyme source.

DNP was inactivated by heating at 65°C for 10 minutes in 0.14 M NaCl-0.01 M citrate. The salt extract from nuclei or DNP derived from 1 gm of liver was incubated with inactivated DNP from 1 gm of liver at 37°C for 15 minutes in 0.1 M NaCl, 25 mM phosphate buffer, pH 8. To prevent precipitation of the extract with the DNP, the reaction was not stopped with TCA, but the DNP spun down and histones

extracted and dried as usual. Where isolated "arginine-rich" calf thymus histones were used as a substrate, the incubation reaction was stopped by precipitation with 15% TCA (since isolated histones are soluble in the medium) and the resulting precipitate was dissolved in 1 M hyamine and counted. Extracts alone and inactivated extracts plus substrate were used as controls.

Extraction of acetylating enzyme from rat liver DNP

Since previous extraction procedures were effective for the isolation of acetylating enzymes from rat liver nuclei, but not from DNP, the possibility was considered that these enzymes represented cytoplasmic contaminants and were not the acetyltransferase in the chromatin complex. Therefore, an attempt was made to extract the acetylating activity from the DNP complex using a method which had been proven to be successful in the isolation of RNA polymerase.[154] Earlier studies on the isolation of RNA polymerase had revealed that this enzyme is tightly bound to the chromatin complex, similar to the chromatin acetyltransferase. In studies on endogenous RNA polymerase activity the crude chromatin was consequently often referred to as the "aggregate enzyme."[155]

Nuclei and DNP from 12 gm of rat liver were gently homogenized in 12 ml of 15 mM phosphate buffer, pH 8, 0.5

mM ethelenediamine tetraacetic acid (EDTA), and 1.0 mM 2-mercaptoethanol. This mixture was incubated at 30°C for 50 minutes with gentle shaking. In the original polymerase extraction procedure a tris-phosphate buffer, pH 8.8, was used,[154] but since phosphate buffer had been shown to be much more favorable to the DNP catalyzed transfer of acetate from acetyl-CoA to histones than tris buffer, the phosphate buffer system was substituted. After the extraction, the mixture was centrifuged at 115,000 x g for 40 minutes and the supernatant served as the enzyme source. One ml of this supernatant contained the enzyme extracted from DNP isolated from 1 gm of rat liver. One ml of enzyme solution was incubated in the presence of acetyl-^{14}C-CoA either with inactivated (by heating at 65°C, 10 minutes) DNP from 1 gm of rat liver or 0.5 mg of isolated "arginine-rich" calf thymus histones. The enzyme was also added back to DNP from 1 gm of liver which had been extracted by the above procedure, i.e., to the 115,000 x g precipitate, in an attempt to restore the preparation to full activity. The final volume was 2 ml and contained 0.25 mM EDTA, 0.5 mM 2-mercaptoethanol, 33 mM phosphate buffer, pH 8, 12 mM NaCl, and 0.01 μc of acetyl-^{14}C-CoA (spec. act. 56 mc/mM). After 15 minutes at 37°C, the fractions containing isolated histones were cooled and made 15% with respect to TCA, and

were filtered on millipore filters. The filters were dried in a hot air oven, dissolved in Bray's scintillation fluid, and counted. The samples containing DNP were cooled rapidly and centrifuged to prevent co-precipitation of the enzyme in 15% TCA. The histones were extracted from the sediment as described above.

Method for Determining the Extent of O-Acetylation of Free Histones and that Occurring in DNP

It is possible to estimate the degree of O-acetylation occurring in proteins by a method devised by Narita.[156] In this procedure one takes advantage of the lability of the O-acetyl bond in the presence of hydroxylamine. Histones or DNP prelabeled with radioactive acetyl-CoA can therefore be incubated in the presence of hydroxylamine. The degree of O-acetylation can subsequently be estimated by measuring the amount of radioactivity released as a result of this treatment.

Pogo et al. have shown that the acetate incorporated in the f_{2a1} histone fraction of regenerating rat liver was stable to treatment with 2 M hydroxylamine,[110] indicating that this fraction does not contain O-acetyl groups. Other studies on the site of acetylation of histones in calf thymus nuclei f_{2a1} fraction showed that acetylation only takes place at the epsilon-amino group in lysine.[93] The

f_3 fraction of regenerating rat liver, however, showed a release of 55% of the acetyl groups as a result of hydroxylamine treatment,[110] which agrees with the work of Nohara *et al.* who reported considerable O-acetylation of the f_3 fraction by pigeon liver enzymes *in vitro*.[117] In the case of calf thymus, all of the acetate incorporated in the f_3 fraction upon incubation of nuclei in the presence of ^{14}C-acetate was recovered as epsilon-N-acetyllysine after enzymatic digestion and ion exchange chromatography.[94] Thus, there is, at least in calf thymus, no evidence for the formation of O-acetyl linkages under these conditions.

Since it may be expected that an enzymatically catalyzed reaction will show greater specificity of acetylation and therefore perhaps a lower level of O-acetylation than that occurring spontaneously, DNP catalyzed acetylation in the presence of acetyl-CoA was compared with that occurring spontaneously by free histones with respect to O-acetylation. Conditions were selected to yield a similar amount of radioactivity by either process. Therefore, for DNP catalyzed acetylation, DNP equivalent to 1 gm of liver was incubated in the presence of 0.01 μc of acetyl-^{14}C-CoA, 12 mM NaCl and 25 mM phosphate buffer, pH 8, for 15 minutes at 37°C. The reaction was stopped with 5% TCA, and the histones were dried with acetone-1% HCl and ether.

For the hydroxylamine test, 0.45 ml of hydroxylamine solution (pH 6.4) containing 3 volumes of 40% hydroxylamine hydrochloride and 2 volumes of 3.5 N NaOH were added to 0.3 ml of 0.1 N acetate buffer, pH 5.4, and 0.3 ml of H_2O containing 0.3-0.5 mg of ^{14}C-acetylated histones obtained as described above. After standing at room temperature for 5 hours the histones were precipitated with 15% TCA, centrifuged after 4 hours, and dried in acetone-1% HCl and ether. The percent O acetylation was determined by counting the radioactivity released in an aliquot of the 15% TCA supernatant, whereas the extent of N-acetylation was deduced from the radioactivity remaining in the histones after the hydroxylamine treatment.

Determination of Incorporation of Radioactive Acetate into the Various Histone Fractions

Fractionation according to Johns[157]

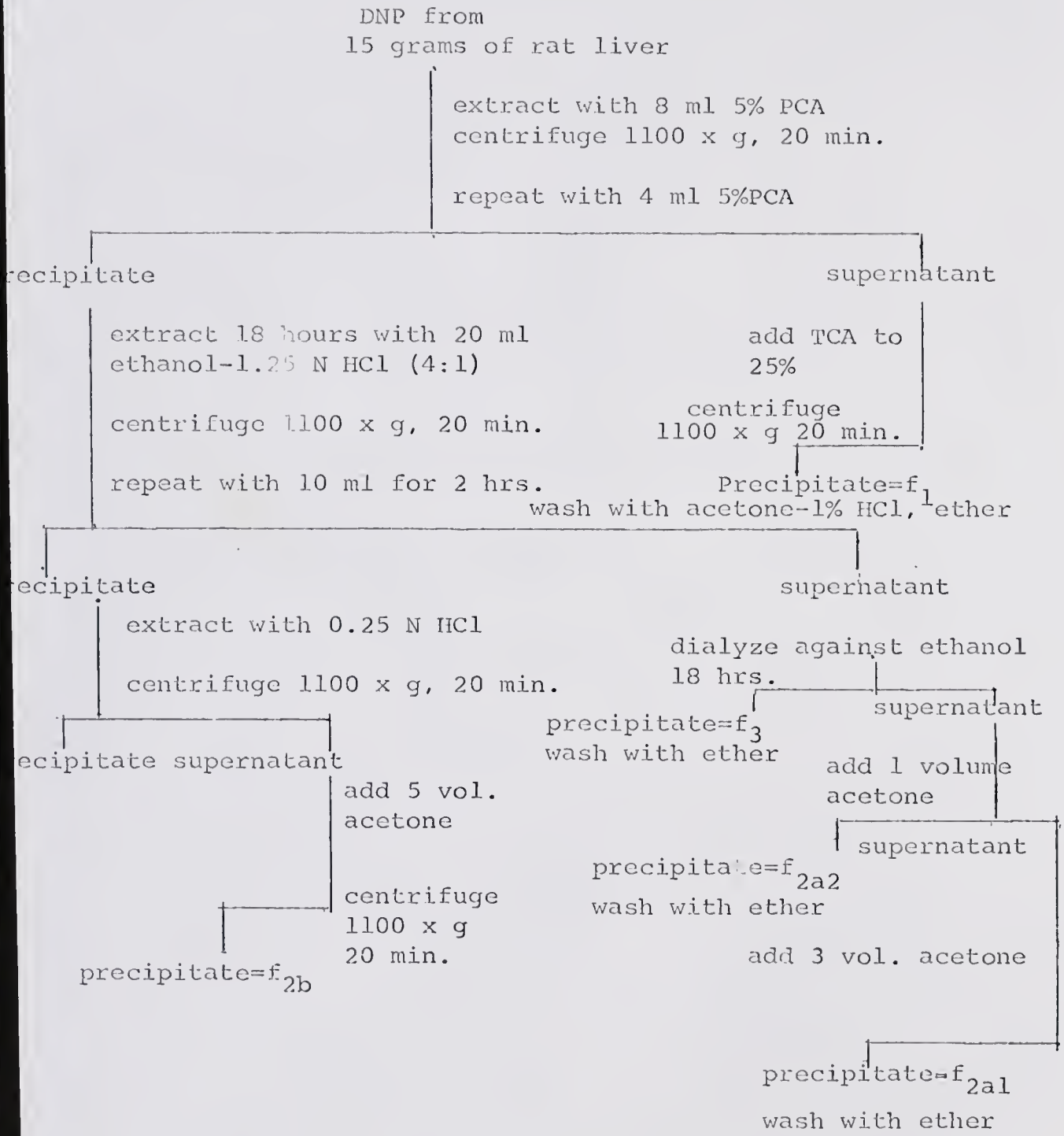
Since it has been observed by others that acetylation of histones occurs primarily in the arginine-rich (f_{2a1} and f_3) fractions (see Introduction), it was deemed important to compare this labeling pattern with that of histones acetylated *in vitro* in DNP. Such a comparison would determine how closely the *in vitro* reaction approximated the *in vivo* situation. Therefore, histones which had been acetylated were separated into the five major fractions according to

Johns and their specific activity determined.[157] These fractions were examined by acrylamide gel electrophoresis to determine the purity of the separation.

For this experiment, DNP was obtained as usual from 15 gm of rat liver and incubated at 37°C for 15 minutes in 5 gm samples containing 10 ml total volume per sample of 12 mM NaCl, 25 mM phosphate buffer, pH 8, and 0.1 μc acetyl-^{14}C-CoA. After the incubation, the reaction was stopped by centrifugation and the unbound label was rinsed out by washing once with a large volume of saline-citrate. Then, the very lysine-rich (f_1) fraction was extracted from the DNP with 8 ml of 5% perchloric acid (PCA), and centrifuged at 1100 x g for 20 minutes. This procedure was repeated once with 4 ml of 5% PCA. This fraction was precipitated from the combined supernatants with 25% TCA, dried with acetone-1% HCl, and ether. The residual DNP was extracted for 18 hours with 20 ml of ethanol-1.25 N HCl (4:1), with stirring, and centrifuged at 1100 x g for 15 minutes. This extraction was repeated once with 10 ml of ethanol-HCl (4:1), for 2 hours. After centrifugation the combined supernatants contained the arginine-rich (f_{2a} and f_3) fractions, whereas the moderately lysine-rich (f_{2b}) fraction remained still bound to the residual DNP. The supernatants containing the f_{2a} and f_3 fractions were dialyzed against

ethanol for 18 hours, which causes the f_3 fraction to precipitate, leaving the f_{2a} fraction in solution. After centrifugation, the f_{2a} histones were further separated by the addition of an equal volume of acetone. This caused the precipitation of f_{2a2}. After centrifugation f_{2a1} was precipitated from the supernatant with 3 volumes of acetone. The precipitates were dried in ether. The moderately lysine-rich (f_{2b}) fraction was then extracted from the residue with 0.25 N HCl, and precipitated with 5 volumes of acetone, and dried in ether.

CHART 2: OUTLINE OF SEPARATION OF HISTONE FRACTIONS



Electrophoresis of histone fractions

The histone fractions obtained according to Johns were checked for purity by polyacrylamide gel electrophoresis in a vertical electrophoresis apparatus from the E. C. Apparatus Co., Philadelphia, Pennsylvania. Acid gel conditions, designed for the resolution of basic proteins,[158] were used as follows:

Gel and Sample Buffer: Tris 0.12 M adjusted to pH 2.9 with citric acid.

Electrode Buffer: Glycine 0.37 M, adjusted to pH 4.0 with citric acid.

Sample Solvent: Tris-citric acid buffer pH 2.9 containing 6 M urea. The sample solvent was saturated with sucrose to facilitate the settling of the sample in the slots.

Polymer Solution: 12% Cyanogum-41 in tris-citric acid buffer, pH 2.9, containing 3 M urea. Total volume was 150 ml for each gel.

Catalyst: 0.1% ascorbic acid, 0.0025% ferrous sulfate, and 0.02% H_2O_2.

Besides the addition of sucrose to the sample solvent, the only modification of the procedure as originally designed was that the H_2O_2 concentration was dropped from 0.03% to 0.02% to increase the polymerization time long enough to pour the gel.

After mixing the polymer solution, the ascorbic acid and ferrous sulfate were added with stirring. Immediately

after mixing, the H_2O_2 was added and the gel was poured. The electrophoresis apparatus was precooled before pouring the gel by running water at 8°C through the circulation system. This was done to prevent contraction of the gel upon polymerization. Since the gel hardened within a few minutes, it was necessary to remove any bubbles that formed immediately. After about 10 minutes the gel had sufficiently hardened to fill the apparatus with 2 liters of the electrode buffer. It was only after this step that the teflon slot former could be easily removed.

Samples of 15 µl containing 30 µg of protein were placed in each slot and ran at 250 v., 8°C, for 4 hours. It was found in preliminary experiments that no pre-run was necessary using these gel conditions. Staining of the gel was done in 0.2% Amido Schwartz, 7% acetic acid, and 40% ethanol, for 20 minutes, and destaining accomplished electrophoretically by a destainer from E. C. Apparatus Company.

RESULTS

In early experiments the *in vitro* acetylation of histones by isolated nuclei was studied. These nuclei were purified by centrifugation through 2.1 M sucrose as described in Materials and Methods. In the course of these investigations it was discovered that if these purified nuclei were washed with isotonic saline, the resulting DNP preparation possessed an acetylating activity approximately 3 times that of the nuclei. This was an indication that perhaps an acetylating enzyme responsible for the transfer of acetate from acetyl-CoA to histones was present in or at least closely associated with the chromatin complex. Since other laboratories had only reported the isolation of acetylating enzymes from whole tissue or nuclei,[117-119] this finding stimulated interest to characterize this reaction more carefully. An enzyme obtained from the DNP complex had less chance of being a cytoplasmic contaminant than those isolated from nuclei, and, in view of its localization in the chromatin itself, took on more meaning in view of the possibility that histone acetylation plays a role in chromosomal function, e.g., the control of gene expression.

To verify that the acetylation reaction was not due to cytoplasmic contamination, DNP was also prepared from nuclei isolated in detergent. This procedure removes the outer nuclear membrane. DNP prepared from these nuclei was found to possess an acetylating activity approximately equivalent to that of DNP obtained from nuclei isolated by the usual procedure. This would support the contention that the acetylating activity observed in DNP prepared from nuclei by the usual procedure is not of cytoplasmic origin.

Further support for this was obtained from experiments in which DNP isolated according to the usual procedure was further purified by centrifugation at 22,000 rpm for 3 hours in the Spinco S. W. 25 head in 1.7 M sucrose. Marushige and Bonner have shown that rat liver chromatin purified by this procedure is characterized by a low RNA content,[44] and others have reported that the nonhistone protein contained in this preparation is not a cytoplasmic contaminant, but a real constituent of chromatin.[159] DNP treated in this manner showed acetylating activity two or three times greater than that of the usual preparation. These results confirmed that the acetylation reaction was not due to a cytoplasmic contaminant and was closely associated with the chromatin. Having established the localization of the transferase activity in DNP, the following experiments were

designed to characterize the conditions influencing this reaction, using rat liver DNP as the source for enzyme activity as well as the acetate acceptor.

Conditions Influencing the Acetylation of Histones in DNP

Among the conditions influencing the *in vitro* reaction that were studied were divalent cations, medium components, temperature, concentration of substrates, and pH. These are discussed below in that order.

Divalent cations

Nohara *et al.* has found a Mg^{++} requirement for two pigeon liver fractions which act respectively as acetate activating and transferring enzymes in the acetylation of isolated histones.[117] Gallwitz has suggested that Mg^{++} is only necessary for the activating enzyme since a transferring enzyme which he has isolated from rat liver nuclei does not require it.[118] In view of these findings it was decided to study the effect of several divalent cations on the DNP catalyzed transfer of acetate from acetyl-CoA to histones.

Rat liver DNP washed in 0.075 M NaCl and 0.024 M EDTA (chelates divalent cations), pH 8,[160] was incubated in the presence of 5 mM EDTA or different concentrations of divalent cations. The results indicate a depression in activity at 10 mM Mn^{++} and 8 mM Ca^{++}. The presence of Mg^{++} from 0-10 mM did not seem to affect the activity significantly.

Medium components (Table I)

Since the original incubation medium was designed for

the study of protein synthesis by isolated calf thymus nuclei,[144] it seemed desirable to analyze the effects of the various components of this medium on the acetylation reaction. The results of 1 experiment using 4 samples for each condition is reported in Table I. The term "complete medium" refers to the one described in Materials and Methods, except that phosphate buffer, pH 8, rather than pH 6.75, was used and the divalent cations were omitted. These changes were made on the basis of other experiments to be reported here. Components of the complete medium were omitted and the molarity of the buffer was gradually lowered. The results indicate that the presence of sucrose and glucose is not essential for the DNP catalyzed acetylation of histones, and the optimum concentration of the phosphate buffer was 25 mM. The effect of ionic strength on the reaction mixture was studied by changing the concentration of NaCl in the incubation medium. Between 0.01 M to 0.2 M NaCl there was a slight rise in activity up to 0.1 M NaCl, followed by a decrease at 0.2 M NaCl. This is in agreement with a report by Gallwitz and Sekeris on the acetylation of histones by rat liver nuclei.[115] The optimum conditions for this reaction were therefore assumed to be 12 mM NaCl, and 25 mM phosphate buffer, pH 8, according to the parameters studied.

Temperature (Figure 1)

Figure 1 shows the course of the reaction with time at two different temperatures. The medium for this experiment was as described for the original procedure (Materials and Methods) except that the concentration of acetyl-^{14}C-CoA was 4 times higher. From these data a Q_{10} of about 1.8 could be calculated. Allfrey has reported a Q_{10} of 2.09 for this reaction in nuclei.[126] In general the velocity of enzymatic reactions is doubled for a 10^{o} rise in temperature.

Table I

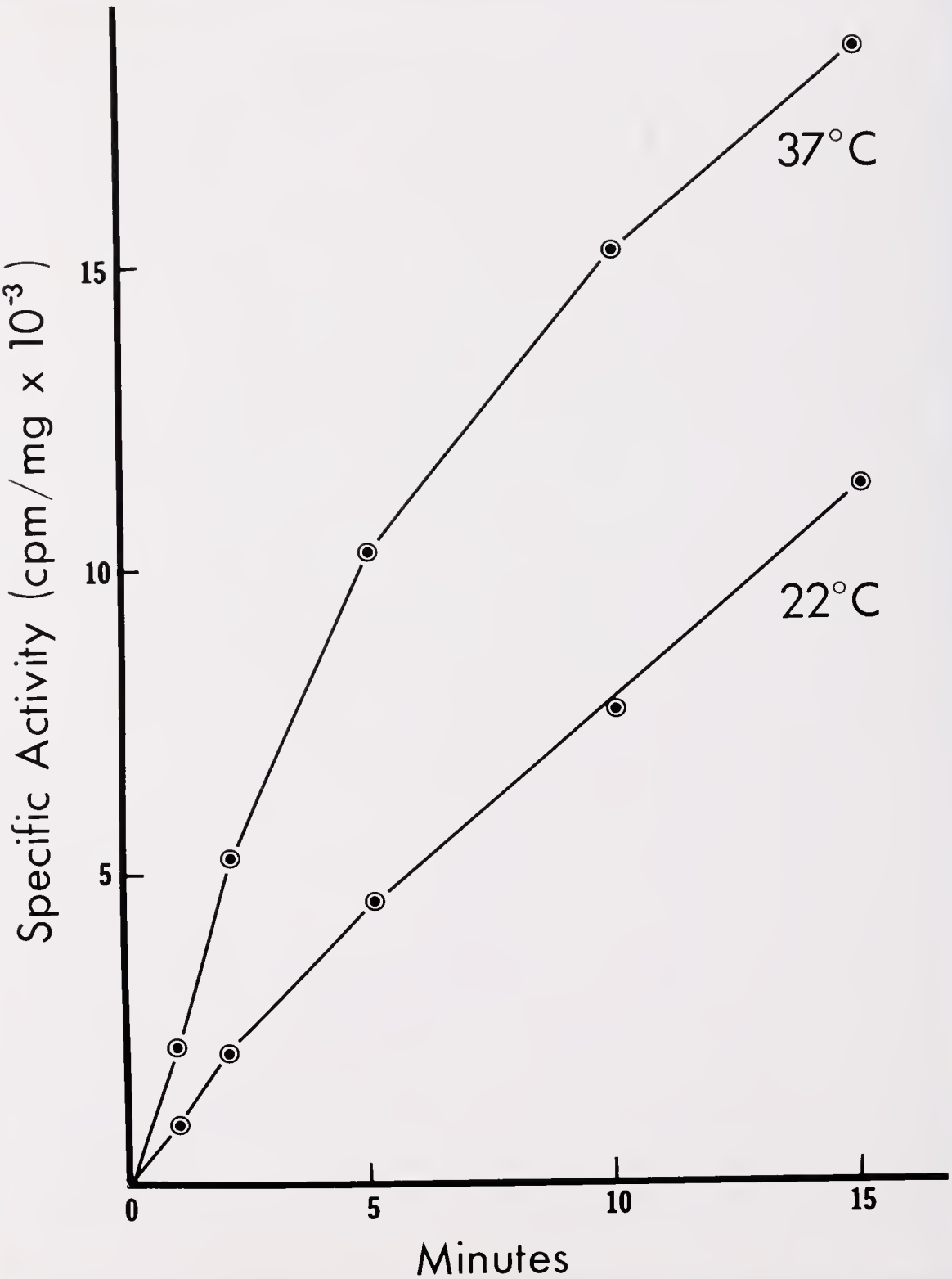
Effect of Various Medium Components on the Transfer of Acetate from Acetyl-CoA to Histones in DNP

Incubation Media	Specific Activity (cpm/mg)	Concentration of Phosphate Buffer
Complete medium	8243 ± 611	25 mM
-sucrose -glucose	9075 ± 910	25 mM
-sucrose -glucose -NaCl	7037 ± 444	25 mM
-sucrose -glucose -NaCl	1729 ± 224	2.5 mM
-sucrose -glucose -NaCl	998 ± 85	0.25 mM
-sucrose -glucose -NaCl	769 ± 76	0.025 mM

The results are expressed as specific activity (counts per minute per mg) of histones plus or minus the standard error.

Figure 1: Effect of Temperature on the Transfer of Acetate From Acetyl-CoA to Histones in DNP.

The results are expressed as specific activity (counts per minute per mg x 10^{-3}) of histones as a function of time.


37°C
22°C
Specific Activity (cpm/mg x 10^{-3})
15
10
5
0
5
10
15
Minutes

Concentration of DNP (Figure 2)

Since in this particular experimental set-up the acetate-accepting substrate and enzyme are both present in the DNP preparation, the histone concentration varies with that of the enzyme when the DNP concentration in the reaction mixture changes. Therefore, it seemed interesting to determine the optimum concentration of DNP for the transfer reaction. Rat liver DNP was incubated at different concentrations in the presence of acetyl-^{14}C-CoA in the usual manner. The results are represented graphically in Figure 2, either as total counts per minute or as specific activity (counts per minute per mg histone). As can be seen, there is a rise in both curves with an optimum concentration of DNP equivalent to 0.3-0.5 mg of histones per 2 ml incubation mixture. Past this point increasing concentrations of DNP affected the total incorporation to a minor degree, but caused a rapid drop in the specific activity of histones.

Concentration of acetyl-CoA (Table II)
and Km determination (Figure 3)

The Km of the transfer reaction for acetyl-CoA was determined by incubating the optimal amount of DNP with increasing concentrations of acetyl-CoA. Results were obtained from 2 separate experiments using isotope solutions containing 3 different specific activities of acetyl-^{14}C-

CoA. The data are given in Table II below. The results were plotted (Figure 3) according to Lineweaver and Burk,[161] giving a Km value of 2.5×10^{-6} M.

Effect of pH in different buffer systems (Figure 4)

In these experiments 25 mM phosphate buffer was substituted by 25 mM tris-HCl buffer or 25 mM glycine-NaOH buffer. The optimum pH for the DNP catalyzed acetylation of histones in glycine-NaOH buffer was found to be 8.5-9.6. The phosphate buffer system appeared the most favorable for the DNP catalyzed reaction, and since pH 8 was the highest pH that can be obtained with this buffer, it represents the optimum buffer conditions for DNP catalyzed acetylation of histones.

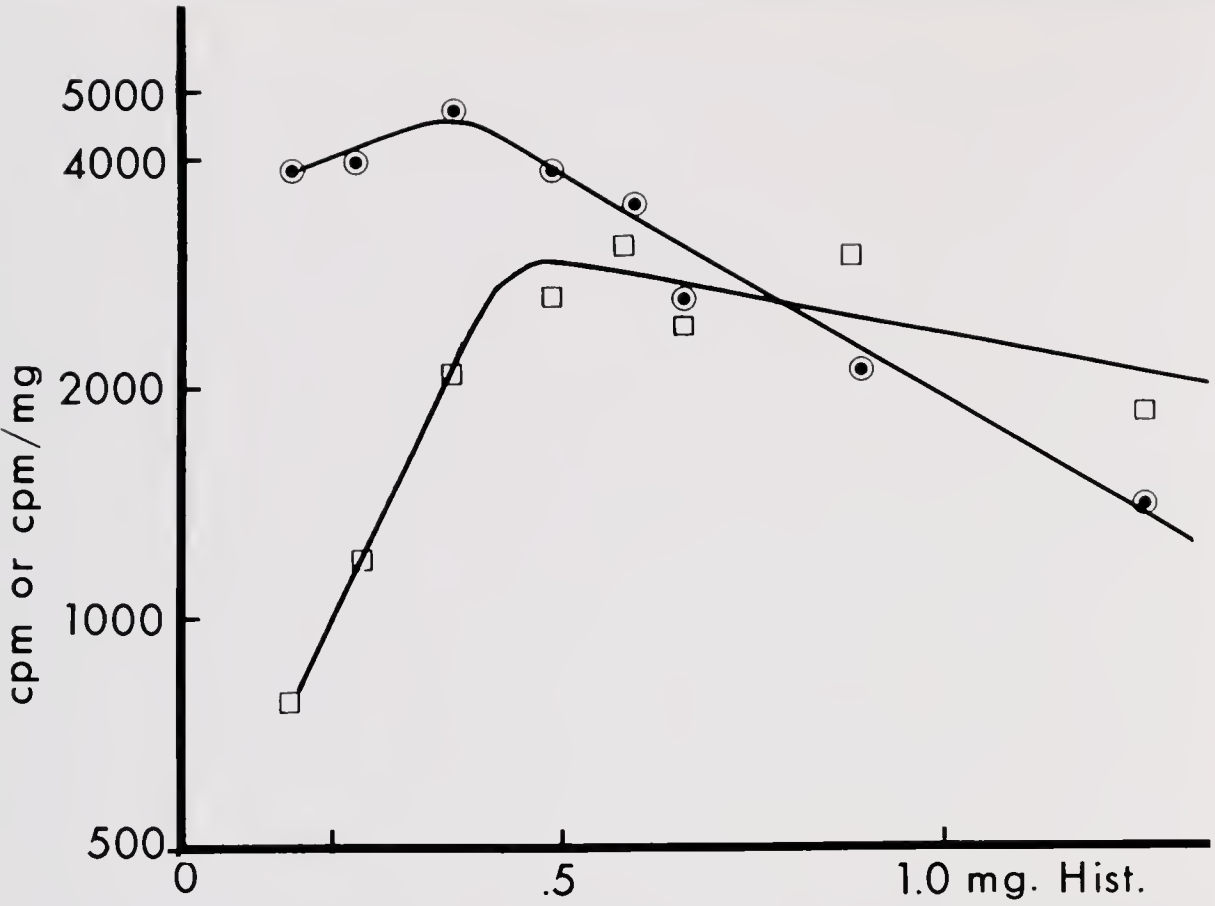


Figure 2: Effect of the Concentration of DNP in the Incubation Mixture on the *In Vitro* Transfer of Acetate from Acetyl-CoA to Histones

DNP was incubated at increasing concentrations in the presence of acetyl-^{14}C-CoA as described in Materials and Methods. Results are expressed as total counts per minute recovered in isolated histones □——□ or as specific activity (counts per minute per mg) ⊙——⊙ of histones. DNP concentrations are expressed in terms of histone content of DNP sample in 2 ml medium.

Table II

Effect of Increasing Concentrations of Acetyl-CoA on the Transfer of Acetate from Acetyl-CoA to Histones in DNP

	Concentration of Acetyl-CoA	Spec. Act. Histones (cpm/mg)	Spec. Act. Acetyl-CoA (cpm/μmole)	μmoles Acetate/ mg Histones
EXP. 1	1.6×10^{-8} M	798		27.7×10^{-6}
	1.6×10^{-7} M	6139		213.2×10^{-6}
	1.6×10^{-6} M	23892	28.8×10^{6}	829.5×10^{-6}
	1.6×10^{-5} M	33892		1176.8×10^{-6}
	1×10^{-4} M	3751		1631.0×10^{-6}
	1×10^{-5} M	3168	2.3×10^{6}	1377.0×10^{-6}
EXP. 2	1×10^{-6} M	21239		212.4×10^{-6}
	0.5×10^{-6} M	29338		293.4×10^{-6}
	0.33×10^{-6} M	18964	1.0×10^{8}	189.7×10^{-6}
	0.25×10^{-6} M	16072		160.7×10^{-6}
	0.20×10^{-6} M	14338		143.4×10^{-6}
	0.166×10^{-6} M	12202		122.0×10^{-6}

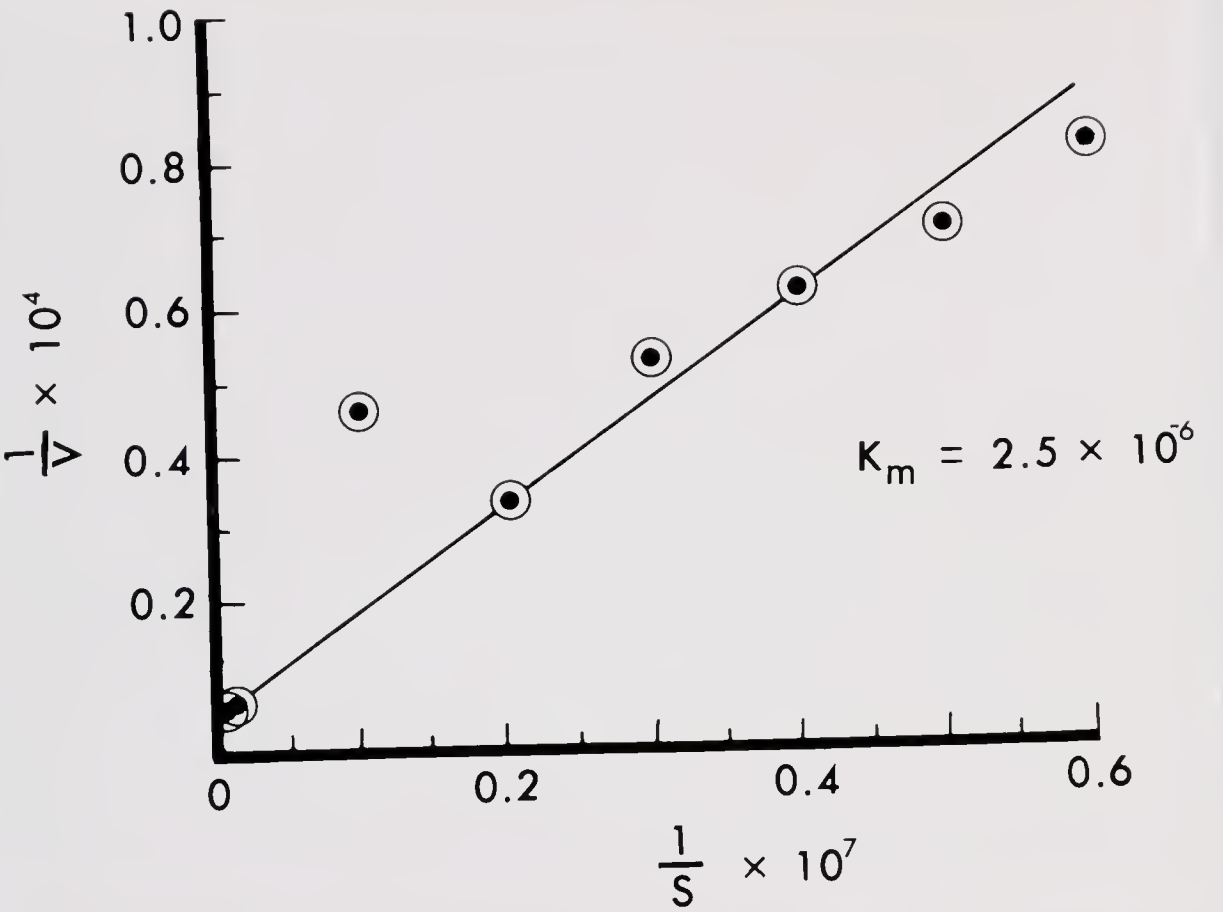


Figure 3: Effect of Increasing Concentrations of Acetyl-CoA on the In Vitro Transfer of Acetate from Acetyl-CoA to Histones in DNP Plotted According to Lineweaver and Burk

Rat liver DNP equivalent to 0.3-0.5 mg histones was incubated in 25 mM phosphate buffer, pH 8, 12 mM NaCl, 0.19 M sucrose and 20 mM glucose in the presence of increasing concentrations of acetyl-CoA as described in Materials and Methods. Calcium and magnesium were omitted. Ordinate: V=µmoles of acetate incorporated per mg histone. Abscissa: S-molar concentration of acetyl-CoA.

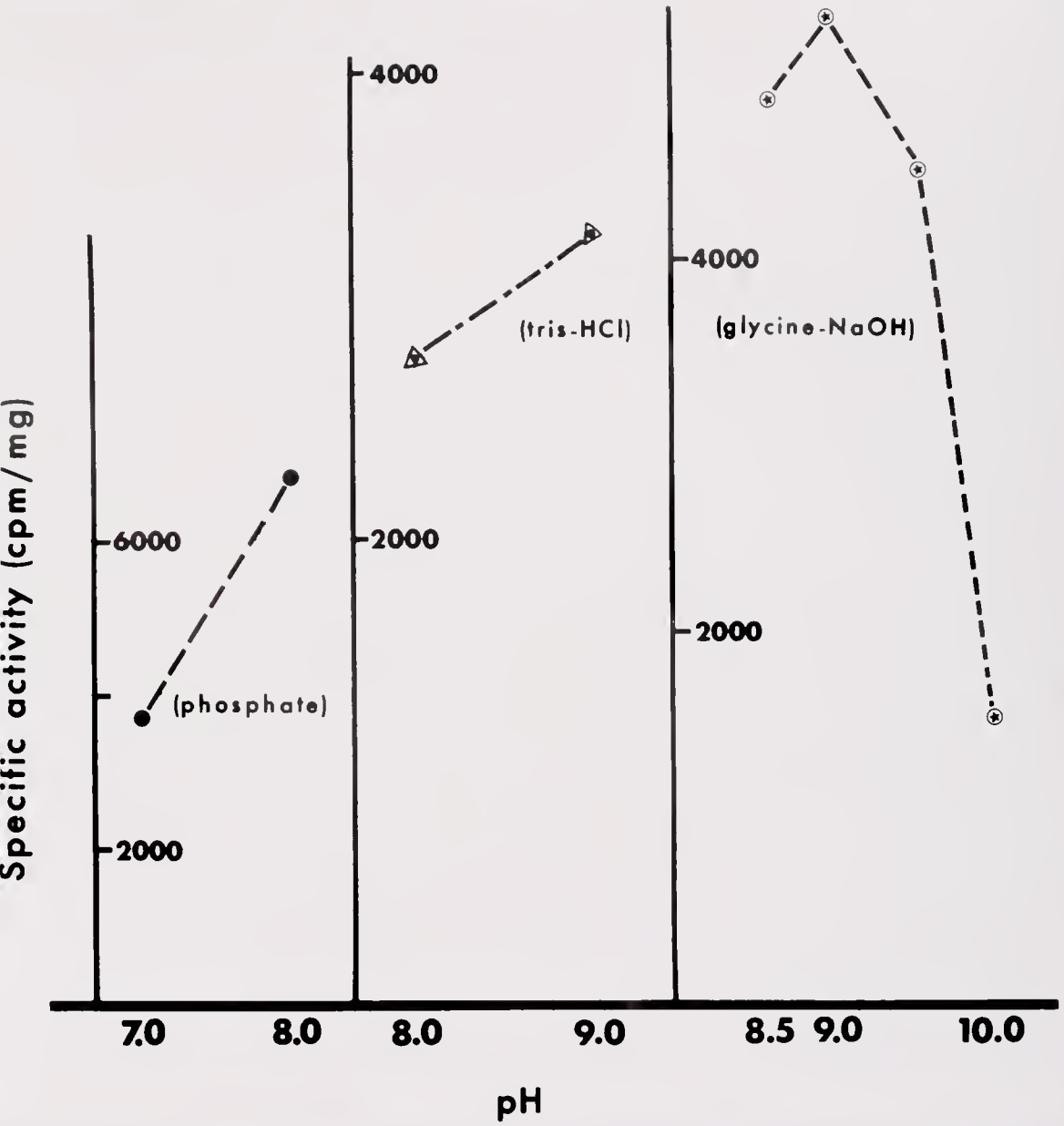


Figure 4: Effect of pH in Different Buffer Systems on the Transfer of Acetate from Acetyl-CoA to Histones in DNP

The results are expressed as specific activity (counts per minute per mg) of histones as a function of pH.

Enzymatic Nature of the DNP Catalyzed Acetylation of Histones

Since it had been reported, and subsequently reconfirmed in this laboratory, that isolated histones will become acetylated spontaneously when incubated in the presence of acetyl-CoA, considerable attention has been given to the possibility that the acetylation of histones occurring within the DNP complex may not be enzymatic.

Inhibition studies (Table III)

The first approach taken to rule out nonenzymatic transfer of acetate from acetyl-CoA to histones was an indirect one. In these studies the DNP complex was treated prior to incubation in various ways to either wash out or extract the acetylating enzyme, or to inhibit its action by methods which are in general considered to be deleterious to enzymes. The effects of these various treatments on the transfer of labeled acetate during subsequent incubation are shown in Table III.

Treatment of DNP in 1 M NaCl causes it to dissociate into histones and DNA.[17, 162, 163] Subsequent dilution of this solution to 0.14 M will result in recombination of histone and DNA. Nonhistone proteins, however, remain in solution. It was found that if liver DNP was subjected to such a treatment the activity decreased to 66% of untreated controls. Although this treatment is expected to remove non-

histone proteins bound within the DNP complex, it appears as though most of the activity remains tightly bound to the DNP.

Recently Johns and Forrester reported that extraction of calf thymus DNP with 0.35 M NaCl removes acidic proteins which have become bound to it during the isolation procedure.[153] An attempt was therefore made to extract the factor responsible for acetylating activity from rat liver DNP with 0.35 M NaCl (see Materials and Methods). The results of these experiments indicate that DNP subjected to such treatment retains 80% of its acetylating activity.

Heating briefly at 65°C, or washing with ethanol, acetone, and ether, practically abolished activity. The histone acetylation in these preparations appeared to be negligible, both under conditions (phosphate buffer) which favor an enzymatic reaction and those (tris buffer) favoring a chemical process (see below). Prolonged incubation of DNP in the regular medium at 0°C and 37°C for different time periods resulted in a decreased activity. Neither the salt extractions, treatment with organic solvents, or heat, changed the histone/DNA ratio of the DNP. It could be argued that the decrease in acetylating activity observed upon incubation of DNP for prolonged periods at different temperatures could be due to an alteration in the histones

rather than a change in an enzyme within the DNP. To check this possibility, "arginine-rich" histones from DNP which had been incubated for 20 hours at 0°C and for 4 hours at 37°C were isolated and their ability to become acetylated spontaneously or enzymatically in the presence of the acetyltransferase (see below) was compared with that of histones isolated from control preparations of DNP. This experiment showed that histones from these pretreated preparations were still capable of becoming acetylated to the same degree as the controls or even higher. These findings argue against the possibility that the histones were degraded or extracted from the DNP complex during these treatments. Therefore the observed decrease in acetylation cannot be explained by a defective substrate, but presumably results from enzymatic denaturation.

Free histones subjected to treatment with organic solvents or heated at 65°C were still capable of becoming spontaneously acetylated. In contrast the acetylation reaction within the DNP is completely abolished by these treatments. These findings suggest that acetylation is somehow prevented in histones which are bound within the DNP complex.

Table III

Effect of Various Conditions on *In Vitro* Transfer of Acetate from Acetyl-CoA to Histones

Pretreatment of Deoxyribonucleoprotein	Percent of Control
Extraction with 1 M NaCl	66.0
Extraction with 0.35 M NaCl	80.0
Organic solvents	4.0
65°C for 5 minutes	1.3
Organic solvents (Incub. at pH 9, 12 mM tris)	1.0
65°C for 5 minutes (Incub. at pH 9, 12 mM tris)	0
Incubated at 0°C for 20 hours in regular medium	70
Incubated at 37°C for 4 hours in regular medium	17

The results are expressed as percent of untreated controls on the basis of specific activity (counts per minute per mg of histones).

Comparison of DNP catalyzed acetylation with spontaneous acetylation of free histones

Buffer Effects (Figure 5)

Comparisons between the pH and buffer conditions influencing the spontaneous acetylation of free histones and that occurring within the DNP were made in an attempt to show that they are, indeed, two different reactions. Figure 5 represents a composite graph which summarizes data from a number of separate experiments in which the spontaneous acetylation of free histones was compared with that occurring within DNP in different buffer systems, at increasing pH. As can be seen the DNP catalyzed reaction is greatly favored by the phosphate buffer system, whereas the spontaneous reaction is more active in tris-HCl buffer. The spontaneous reaction shows an increase with pH in tris buffer, and at pH 9 actually exceeded that occuring in the DNP. Due to the range of the phosphate buffer system, it was impossible to observe the DNP catalyzed reaction under optimum phosphate buffer conditions at a pH higher than 8. Both the spontaneous and the DNP mediated reactions show a pH optimum at pH 8.6-9.6 in glycine-NaOH buffer.

Temperature Effects (Figure 6)

Since most mammalian enzyme reactions show temperature optima close to 37°C, whereas nonenzymatic reactions increase with temperature, it was decided to compare the

spontaneous acetylation of histones with that occurring in DNP at increasing temperatures.

Previous experiments indicated that a phosphate buffer system, pH 8, represented the most favorable conditions obtainable for the DNP catalyzed acetylation of histones and that a tris-HCl, pH 9, buffer represented the optimum condition for the spontaneous acetylation of free histones. Therefore, the transfer of acetate from acetyl-CoA to histones in DNP as well as free calf thymus histones were compared under these conditions at increasing temperature.

The data shown in Figure 6A were obtained at pH 8 in 25 mM phosphate buffer (most favorable conditions for DNP acetylation); those in 6B at pH 9 in 25 mM tris-HCl buffer (most favorable for spontaneous acetylation of free histones). As can be seen, there is a pronounced differential effect of the ionic environment on the two reactions. In both media, however, the qualitative effect of increasing temperature on each of the two processes was the same: the enzymatic reaction in the DNP complex indicating an optimum at 37°C, followed by a sharp decline; while the spontaneous acetylation of isolated histones showed a progressive increase with increasing temperature up to 77°C. This experiment offers further evidence that the acetate transfer occurring in the DNP complex is catalyzed by an enzyme.

Determination of the Extent of O-Acetylation by Hydroxylamine Test (Table IV)

Since most evidence available at present indicates that the acetylation of histones occurs only at the epsilon-amino group of internal lysine residues and at alpha-amino groups of terminal amino acids, it may be expected that O-acetylation often found in *in vitro* systems is an artifact occurring only as a result of spontaneous acetylation. The DNP catalyzed acetylation of histones was therefore compared with that occurring spontaneously by comparing the extent of O-acetylation occurring in either case. The results in Table IV show that 3% of the acetyl-groups of "arginine-rich" histones acetylated by DNP can be released by hydroxylamine treatment, whereas about 28% can be released from "arginine-rich" histones acetylated spontaneously. This suggests a greater degree of specificity occurring in the DNP catalyzed reaction and implies an enzymatic rather than a random spontaneous process.

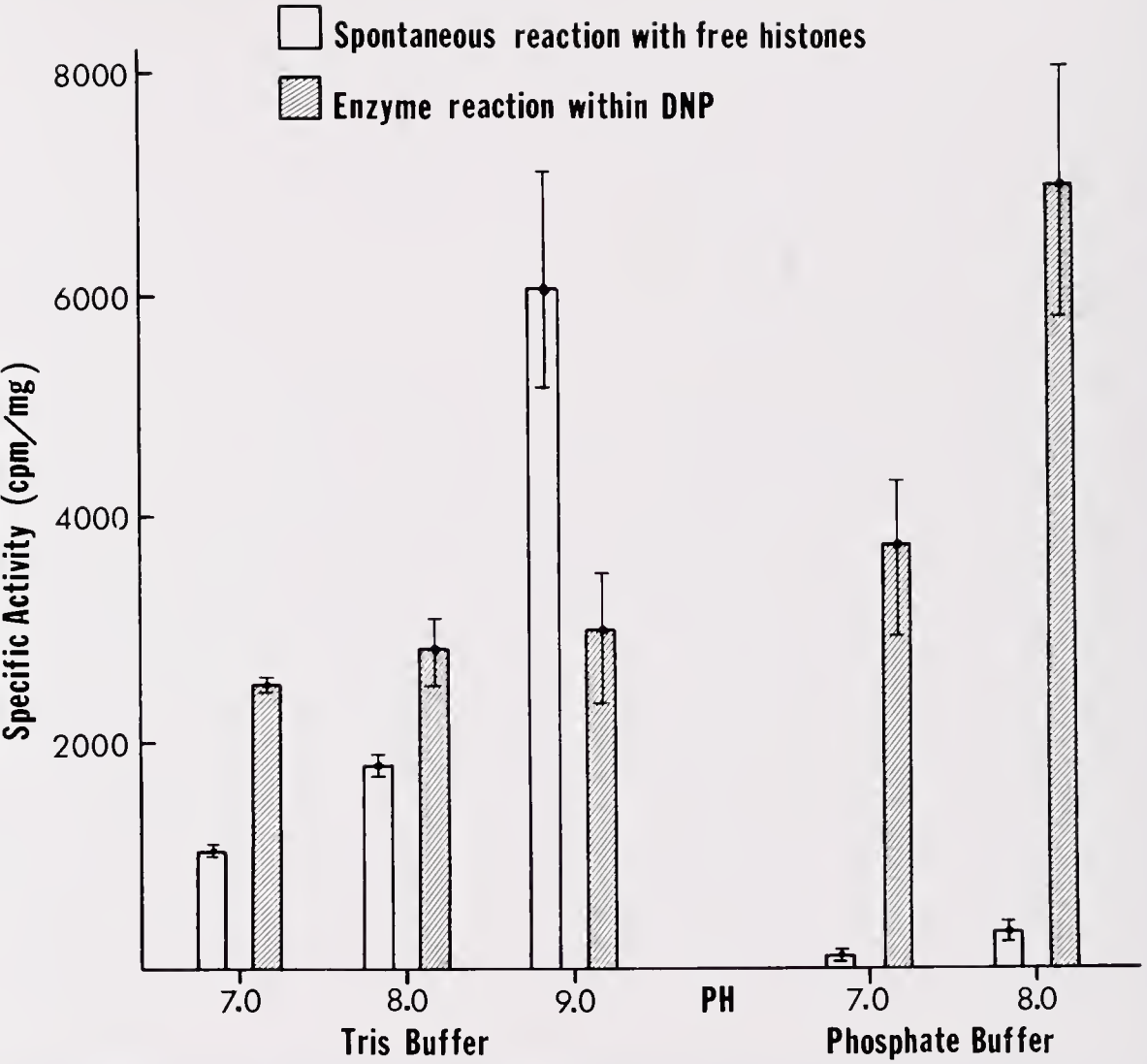


Figure 5: Comparison of the Effect of Increasing pH in Different Buffer Systems on the Spontaneous and Enzymatic Acetylation Reactions

Results are expressed as specific activity (counts per minute per mg) of histones. Range of data is given in brackets.

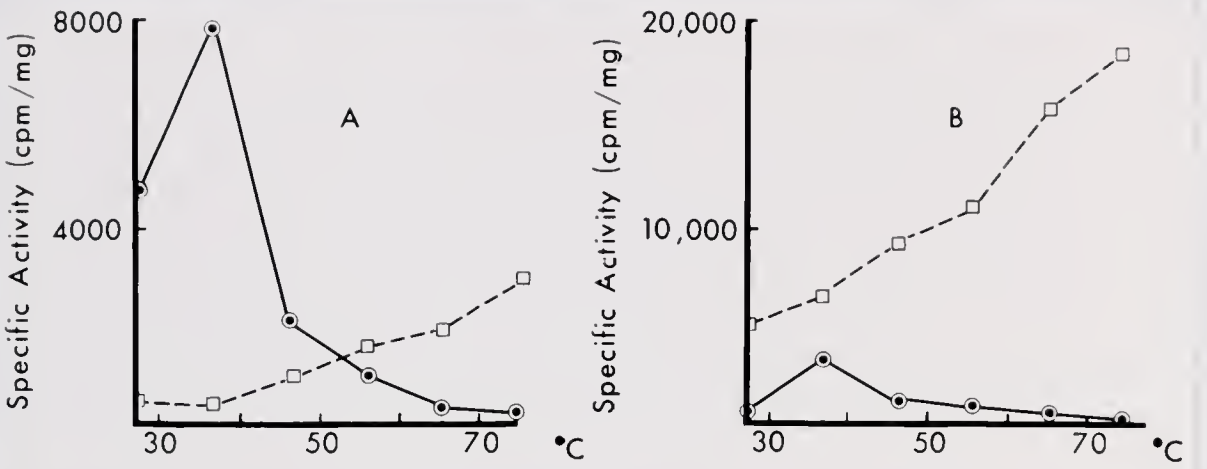


Figure 6: Effect of Temperature on the Transfer of Acetate from Acetyl-CoA to Histones in Rat Liver DNP ⊙———⊙ , and to Free Histones □- - - - -□ in Different Buffer Systems

In Figure 6A, the incubations were carried out in 25 mM phosphate buffer, pH 8; in Figure 6B, in 25 mM tris buffer, pH 9. Calcium and magnesium were omitted in both. Results are expressed as specific activity (counts per minute per mg) of histones.

Table IV

Comparison of the Extent of O-Acetylation in DNP with that Occurring Spontaneously as Measured by Lability to Hydroxylamine

Type of Reaction		Labile Acetyl Group (cpm)	Stable Acetyl Group (cpm)	Percent O-Acetylation
Acetyl-Transferase in DNP	Exp. 1	67	2267	2.8
	Exp. 2	47	1149	3.9
Spontaneous Acetylation of Free Histones	Exp. 1	1340	3994	25.1
	Exp. 2	579	1287	31.0

Results are expressed as total counts per minute. Percent O-acetylation was derived from the amount of label released by the hydroxylamine and the total counts recovered.

Enzyme isolation

Although the evidence reported above suggests the presence of an enzyme within the DNP complex which is responsible for the transfer of acetate from acetyl-CoA to histones, conclusive proof of this hypothesis would be the isolation of the enzyme (acetyltransferase). The results of attempts to this effect are reported below.

Acetylating Enzymes from Rat Liver Nuclei

Acetone powder extracts

The first attempts at isolation followed procedures reported to have been successful for the isolation of histone acetokinases from rat brain and liver nuclei.[118, 151] Following the procedure given in Materials and Methods, it was found that a low activity acetylating enzyme could be isolated from nuclei using this procedure, but not from DNP. In fact, protein determinations of the tris extract of the acetone powder preparation from DNP showed that no protein whatsoever could be extracted by this procedure. When the acetone powder of DNP or nuclei remaining after tris extraction was tested for acetylating activity, it showed about 50-70% of the activity usually observed in normal DNP preparations. This suggests that the enzyme responsible for the reaction in the DNP complex is still tightly bound to the DNP and must therefore be decidedly different from that which is extractable from nuclei.

Saline extracts (Tables V, VI)

Since it has been reported that washing DNP in 0.35 M NaCl removes acidic proteins,[153] saline extracts were made of nuclei and DNP which were examined for acetylating activity. Table V shows the results of 3 different experiments in which these extracts, in quantities comparable to the native preparation, were added back to inactivated DNP. As can be seen, no activity was restored to inactivated preparations by the addition of extracts from DNP, although the addition of extracts from nuclei did restore the acetylating activity to 10% of that of the controls. Table VI shows the activity of salt extracts of nuclei using isolated "arginine-rich" calf thymus histones and polylysine as acetate acceptors. It can be seen that under these conditions this enzyme lacks specificity, as polylysine was acetylated to the same degree as isolated histones.

Table V

Restoration of Acetylating Activity
in Heated DNP by 0.35 M Extracts from Nuclei

Experiment	1	2	3
Control DNP	8505 ± 1340	10997 ± 758	6718 ± 474
65°C inactivated DNP	306 ± 64	292 ± 156	54 ± 46
65°C inactivated DNP and 0.35 M extract of DNP	284 ± 101	171 ± 6	
65°C inactivated DNP and 65°C inactivated 0.35 M extract of DNP	196 ± 36	0	
65°C inactivated DNP and 0.35 M extract of nuclei		1306 ± 27	792 ± 50

Results are expressed as specific activity (counts per minute per mg) of histones plus or minus the standard error.

Table VI

Acetylation of Isolated Histones with a 0.35 M NaCl Enzyme Extract from Nuclei

Incubated Material	Total Counts per Minute
Isolated Histones	131 ± 23
Histones and extract	1677 ± 275 (enzyme extracted from 1 gm liver)
Histones and extract	1199 ± 1075 (enzyme extracted from 0.5 gm liver)
Histones and 65°C inactivated extract	113 ± 26 (enzyme extracted from 0.5 gm liver)
Extract alone	268 ± 4 (enzyme extracted from 0.5 gm liver)
Polylysine	267 ± 48
Polylysine and extract	1288 ± 368 (enzyme extracted from 0.6 gm liver)

Results are expressed as total counts per minute plus or minus the standard error.

Acetylating Enzymes from Rat Liver DNP (Table VII)

A method similar to one used for the isolation of RNA polymerase from the "aggregate enzyme complex" proved to be successful for the extraction of the acetyltransferase activity from DNP.[154] Table VII shows the results of 2 experiments in which the extract containing acetyltransferase activity from DNP from 1 gm of liver was added back to inactivated DNP, free histones, or to DNP which had been extracted by this procedure. In each instance, the substrate contained histones in quantities equivalent to that found in 1 gm of liver. In the case of samples in which isolated histones served as the substrate, the specific activity was calculated by dividing the total counts obtained by the amount of histones added to the reaction mixture. Since there were no histones present in the case of the incubation of the extract alone, this hypothetical figure represents the specific activity calculated by assuming that the standard amount of histones was present. This figure was derived by dividing the total counts per minute in these samples by 0.5 mg of histones.

As can be seen, the extract can only restore activity to heated DNP to 10% of the control level, although about 50% of the acetylating activity appears to be extracted by this procedure. When the extract is added back to DNP

which has been extracted by this procedure, again, only approximately a 10% increase is observed. However, when the extract is added to free histones, there is a high rate of acetylation.

Table VII

Extraction of Histone Acetyltransferase Activity from DNP

Incubated Material	Exp. 1 Specific Activity (cpm/mg)	Exp. 2 Specific Activity (cpm/mg)
Normal DNP	11204 ± 1608	5857 ± 1046
DNP heated at 65°C, 10 min.	172 ± 131	134 ± 82
Heated DNP and extract	1140 ± 75	537 ± 35
"Arginine-rich" histones (0.5 mg)	1142 ± 108	528 ± 47
Extract	474 ± 18*	440 ± 8*
"Arginine-rich" histones (0.5 mg) and extract	9668 ± 335	4445 ± 287
Extracted DNP	6107 ± 228	3523 ± 79
Extracted DNP and extract added back	7013 ± 198	4898 ± 708

The amount of DNP or extract used for each incubation was obtained from 1 gm of rat liver. The results are expressed as specific activity (counts per minute per mg) of histones plus or minus the standard error.

*Specific activity was calculated on the assumption that 0.5 mg of histones was present in the reaction mixture.

Relative acetylation of the various histone fractions (Table VIII) (Figure 7)

After incubation of DNP in the presence of acetyl-CoA as described, the histones were extracted and fractionated according to Johns into the five major histone groups.[157] Table VIII shows the specific activity of each of these fractions. As can be seen, the arginine-rich histones, f_{2a1} and f_3, are the most actively acetylated fractions, with some activity also in the f_{2a2} fraction. The radioactivity of the lysine-rich fractions, f_1 and f_{2b}, was low which correlated with *in vivo* findings.

The purity of these fractions was checked by acrylamide gel electrophoresis and Figure 7 illustrates the patterns observed. As can be seen the f_{2a2} sample shows a band corresponding to the f_{2a1} band and therefore this fraction is probably slightly contaminated with f_{2a1} histones. This could account for some of the activity observed in the f_{2a2} sample, as the f_{2a1} fraction is very highly labeled. Fractions f_3, f_{2a2}, and f_{2b} are not completely separated by this procedure, when all the fractions are combined, but when run separately f_3 and f_{2b} each show only one distinct major band and therefore were considered to be relatively homogeneous.

Table VIII

Acetylation of Various Histone Fractions in DNP

	Fraction	Specific Activity (cpm/mg)
Lysine-rich histones	f_1	557
	f_{2b}	2,909
	f_{2a1}	16,041
Arginine-rich histones	f_{2a2}	8,713
	f_3	12,904

The results are expressed as specific activity (counts per minute per mg) of histones.

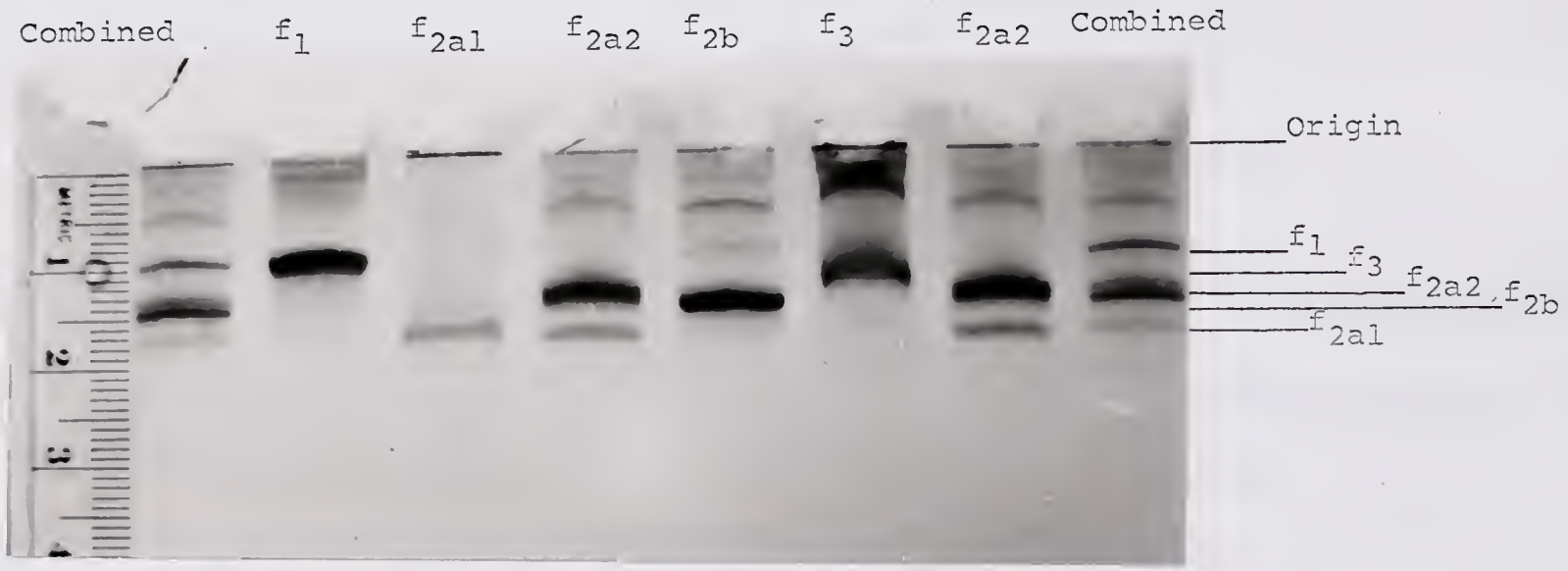


Figure 7: Acrylamide Gel Patterns Obtained with Various Histone Fractions

From left to right the slots contained combined fractions, f_1, f_{2a1}, f_{2a2}, f_{2b}, f_3, f_{2a2}, and combined fractions.

DISCUSSION

Since previous work has revealed that free histones are spontaneously acetylated upon incubation with acetyl-CoA,[118] the possibility arose that the acetylation of histones occurring in DNP might not be enzymatically catalyzed. Therefore, attention has been given to this problem and evidence is provided for the presence of an acetyltransferase bound to DNP which is responsible for this reaction.

Indirect evidence suggesting that the *in vitro* acetylation is catalyzed by an enzyme was provided by experiments in which DNP was pretreated in a way which would be expected to denature a complexed enzyme. Heat or organic solvents, which are generally considered to be inhibitory to enzyme reactions, practically destroyed the acetylating activity of DNP. The fact that after these procedures the histone/DNA ratios were unchanged implies that the physical composition of the DNP complex was the same even after treatment. Therefore, the inhibitory action observed can be attributed to denaturation of an enzyme responsible for the acetylation reaction, especially since similar pretreatment of histones did not affect their spontaneous acetylation. DNP subjected

to heat or organic solvents does not catalyze the acetylation reaction, even under conditions most favorable for a spontaneous transfer of acetate to histones. Apparently, spontaneous acetylation is somehow prevented in histones bound to DNA in the DNP complex. This may have biological implications for the mechanism of gene control in that only an enzymatically catalyzed reaction, capable of a great deal of specificity, rather than a random process, is permitted within the DNP complex.

In the case where DNP showed a loss in acetylating activity as a result of preincubation at 37°C for 4 hours, it could be argued that the histones themselves were degraded by this treatment and thereby were incapable of becoming acetylated. However, if histones are isolated from thusly treated DNP, they are still capable of becoming spontaneously acetylated or acetylated in the presence of a subsequently prepared extract containing acetyltransferase. These histones as well as the acetyltransferase are therefore relatively unchanged with respect to their ability to become acetylated.

Although the above mentioned pretreatments of DNP, especially heating at 65°C, did not change the composition of the DNP with respect to histone/DNA ratios or the ability of the subsequently isolated histones to become spontane-

ously acetylated, it could be argued that the structure of the complex was altered in such a way as to prevent a non-enzymatic transfer of acetate causing the observed inhibition. This seems unlikely, however, as it has been demonstrated that the DNA of liver chromatin is stabilized against heat denaturation as compared with deproteinized liver DNA.[44] The temperature of half-melting (T_m) is 68°C in the case of naked DNA, but is increased to 81°C for chromatin. Preparations of DNP heated at 65°C according to the methods described herein, therefore, probably represent a native structure.

Comparisons between acetylation of complexed histones in DNP and that of free histone occurring spontaneously revealed that, although the pH optima of the two reactions was the same, the DNP reaction was much more efficient in a phosphate buffer system, whereas the spontaneous reaction was greatly favored by a tris-HCl buffer. Furthermore, the DNP catalyzed reaction showed a marked temperature optimum of 37°C, which is common for mammalian enzyme reactions, while the spontaneous reaction rate increased with increasing temperature. These data would seem to indicate that one is dealing with two completely different processes.

Another difference between the acetylation of histones in DNP and that of free histones was the degree of O-acetyla-

tion. Acetyl groups of "arginine-rich" histones acetylated by DNP showed a much greater degree of stability to hydroxylamine treatment than those acetylated spontaneously. This indicates that most of the acetate is N-linked, which correlates with findings by others on the acetylation of histones by calf thymus nuclei.[93, 94] Although findings by others on regenerating rat liver showed 55% O-acetylation for the f_3 fraction, acetate incorporation in the f_{2a1} fraction was stable to hydroxylamine treatment.[110] Using the same procedure Gallwitz and Sekeris also found 35% O-acetylation among the acetate groups of the f_3 fraction acetylated *in vitro* in rat liver nuclei, but none in the other fractions.[115] These findings are probably an exaggerated estimate of the degree of O-acetylation since Perlmann has reported that the epsilon-N-acetyl groups of the lysine residues of pepsinogen are also split by hydroxylamine treatment.[164] Furthermore, direct chromatographic analysis of tryptic and pronase digests of labeled histones according to Gershey *et al.* indicates that 80% of the radioactivity was present as epsilon-N-acetyllysine.[93]

Using similar methods, Vidali *et al.* have also reported that the radioactive acetate of isolated histones acetylated in calf thymus nuclei *in vitro* can be recovered as a single chromatographic peak which was identified as epsilon-N-

acetyl lysine.[94] Since the latter represent more careful and reliable studies, it is probably safe to assume that N-acetylation is the physiological mode of acetylation which occurs *in vivo*, whereas O-acetylation is an artifact of the spontaneous reaction. The results reported in this study show only a 3% release of acetate from "arginine-rich" histones acetylated by DNP upon exposure to hydroxylamine.

The conclusion that the DNP catalyzed acetylation of histones approximates the natural process and does not represent an artifact is supported by the finding that the *in vitro* pattern of labeling, in which the most actively acetylated fractions are f_{2a1} and f_3, is similar to that obtained *in vivo* after administration of ^{14}C-acetate.[110] Therefore, in studies using whole animals, nuclei or DNP, only the arginine-rich fractions are capable of becoming acetylated whereas the lysine-rich (f_1 and f_{2b}) are not.

A further similarity between the acetyltransferase acting in DNP with acetyltransferring enzymes described by others is the lack of a Mg^{++} requirement. Previous investigations have shown that Mg^{++} is required for the transfer of acetate to acetyl-CoA by a fraction from an acetone powder extract from pigeon liver,[117] but not for the transfer of acetate from acetyl-CoA to p-aminobenzoic acid.[165] Similarly Gallwitz showed that there is no Mg^{++} required for

the transfer of acetate from acetyl-CoA to histones by an acetokinase isolated from rat liver nuclei.[118] Choline acetyltransferase activity from pigeon and sheep liver is also not affected by addition of Mg^{++} up to 30 mM.[166]

Another similarity between the histone acetyltransferase reported here and other acetyltransferases concerns the pH requirements. Arylamine acetyltransferase, showing a wide variety of specificities, has a broad pH optimum from pH 6-9.5 when p-nitroaniline is the substrate.[167] When histamine is the substrate, the reaction falls off sharply below pH 8.5, which is similar to the DNP acetyltransferase. Gallwitz has claimed that an acetokinase isolated from rat liver nuclei is inactive at pH 9 although he has found an increase in acetylating activity on raising the pH 7.5 to 9.0 in whole nuclei.[113, 118] He attributes the incorporation of acetate into histones at pH 9 in nuclei to a spontaneous process, but his data show that heat inactivates 90% of this reaction, suggesting that it is primarily enzymatic.

The most conclusive evidence for the demonstration that this reaction is enzymatically catalyzed was the extraction of acetyltransferase activity from the DNP complex. This was achieved using a method found successful for the extraction of RNA polymerase from chromatin.[154] After the

extraction, 50% of the acetylating activity still remained bound to the DNP complex. However, when the extract from an equivalent amount of DNP was added back to heat inactivated or extracted DNP, only a 10% increase was observed. This difference represents a loss which is equal to inactivation of 40% of the total enzyme activity. Another possibility is that the enzyme in the natural state is structurally complexed with the DNP in a certain way. After extraction of the enzyme, it cannot regain this native state by readdition to the DNP. The same amount of extract was able to acetylate free histones actively. It is possible that acetyltransferase as it occurs in the DNP complex, in contrast to the free state, is restricted in its action. Perhaps this restriction may play a role in the regulation of genetic activity by allowing only specifically selected sites of the histones to become available for acetylation. This would in turn affect the DNA-histone interaction and could reduce repression of transcription in certain areas of the genome.

In conclusion, this study demonstrates the presence of an enzyme, histone acetyltransferase, present in and closely associated with rat liver chromatin which is responsible for the transfer of acetate from acetyl-CoA to the lysine residue of "arginine-rich" histones. This finding is con-

sistent with the hypothesis that histone acetylation may be biologically significant as a mechanism involved in the control of gene activity.

ADDENDUM

Preliminary attempts have been made to correlate the acetyltransferase activity with some biological phenomena.

Comparison of Acetylating Activity in Novikoff Hepatoma and Liver DNP (Table IX)

One of these investigations involved a comparison between the acetyltransferase activity of DNP from Novikoff hepatoma with that of liver. *In vivo* experiments from this laboratory had shown that the turnover of acetyl-groups in Novikoff hepatoma histones was very slow relative to that of normal liver or other tissues.[168] It was therefore decided to compare the activity of acetyltransferase in tumor DNP with that in normal liver DNP. Table IX shows the results of 3 separate experiments in which the *in vitro* rate of acetate incorporation into histones from liver and Novikoff hepatoma DNP were compared, and one in which nuclei were used rather than DNP. Although the specific activity of the tumor histones was about half of that of liver histones, the magnitude of the difference did not seem adequate to account for the almost complete lack of turnover of acetyl groups by Novikoff hepatoma observed *in vivo*.

A corrollary to this finding was the observation that DNP possessed an acetylating activity about 3 times that of nuclei. Therefore, it was hypothesized that perhaps a deacetylating enzyme was present in the nucleoplasm and differences in the activity of this enzyme in tumor as compared with liver could account for the *in vivo* observed differences.

Table IX

In Vitro Uptake of Acetate into Histones: Comparison of Liver and Novikoff Hepatoma

Experiment	Uptake of ^{14}C-Acetate as cpm/mg Histone			
	Nuclei		DNP	
	Tumor	Liver	Tumor	Liver
1	1052 ± 47	2213 ± 77	3031 ± 531	7132 ± 259
2	---	---	3107 ± 141	6307 ± 231
3	---	---	3062 ± 63	4334 ± 152

Incubations were conducted as described in Materials and Methods. The results are expressed as specific activities (counts per minute per mg) of histones plus or minus standard error.

Deacetylating Activity in Nuclear Extracts (Table X)

Inoue and Fujimoto had found deacetylating activity in a 0.14 M NaCl extract of calf thymus.[122] Therefore, a similar extract was made of rat liver nuclei and tested for deacetylating activity.

To measure deacetylation, ^{14}C-acetate labeled DNP was prepared as follows: DNP from 5 grams of rat liver was isolated as described and incubated for 15 minutes at 37°C in a final volume of 10 ml, containing 12 mM NaCl, 25 mM phosphate buffer, pH 8, and 0.5 μc (1×10^{-6}M) acetyl-^{14}C-CoA. The reaction was stopped by rapid cooling and centrifugation (10 min., 2000 x g). The precipitates were washed an additional 2 times with saline-citrate containing 10^{-5}M unlabeled acetyl-CoA, and 1.7×10^{-6} M acetic acid designed as carriers to remove any label not covalently bound. ^{14}C-acetyl labeled "arginine-rich" histones were prepared from the thusly isolated DNP according to the procedure described above. A crude nuclear deacetylating enzyme extract was prepared by homogenizing nuclei from 16 grams of rat liver in 3 ml of 0.14 M NaCl and centrifuging at 8,000 x g for 15 minutes. 0.3 ml of this supernatant containing 1.5-3.0 mg of protein was added to 0.3 ml of 50 mM tris-HCl buffer pH 7.3 containing 0.1-0.2 mg of the labeled histones and the mixture was incubated at 37°C for

20 minutes. The reaction was stopped by the addition of 0.1 ml of 0.1 N HCl containing 5 μmoles of carrier acetic acid. The acidified incubation media were extracted with ethyl acetate and their radioactivity determined by liquid scintillation counting.

Table X shows the results of such an experiment, which clearly demonstrates the presence of a deacetylating enzyme in the nuclear sap. The results indicate that exposure to the crude deacetylating enzyme released 72% of the labeled acetate from free histones and 13% from DNP containing an equivalent amount of histones. It is interesting to note that less acetate is removed from DNP than from free histones. This is a parallel to the finding that the isolated acetyltransferase is also more active in the transfer of acetate to free histones than when they are bound to DNA within the DNP complex. This suggests a restrictive action by DNA and/or the nonhistone proteins in the DNP influencing the availability of the sites for acetylation and deacetylation. Perhaps this may be related to transcriptional control mechanisms and the activity of RNA polymerase.

When the activity of nuclear extracts containing deacetylating enzymes (same amount of protein in each) from liver and tumor were compared preliminary results suggested that the deacetylating activity is much less in the tumor

Table X

Deacetylation Activity in Nuclear Sap Extracts

	Acetyl-^{14}C-DNP	Acetyl-^{14}C-Histones
Radioactivity of starting material (cpm)	12668	10798
cpm extractable with ethyl acetate from the incubation medium after incubation:		
1. in absence of nuclear sap extract	884	9
2. in presence of heat inactivated (10 min., 70°C) nuclear sap extract	970	17
3. in presence of nuclear sap extract	2599	7859

Results are expressed as total counts per minute.

than in liver. As these studies were being conducted, Libby published a report showing that whole nuclei from liver incubated with acetylated histones prepared from calf thymus could release acetate about 7 times faster than nuclei from Novikoff hepatoma.[169] Libby's experiments seem to support the findings reported here and perhaps offer an explanation for the lack of acetate turnover observed *in vivo* in Novikoff hepatoma.

Effects of Treatments Known to Increase RNA Synthesis on the Acetylation of Histones in DNP

Acetylation of histones has been implicated as a mechanism for influencing gene expression. Moreover, others have shown that an increase of *in vivo* acetylation precedes an increase in RNA synthesis (see Introduction). Therefore, the effect of treatments known to stimulate RNA synthesis *in vivo* was studied on *in vitro* acetylation of histones in DNP.

The most extensive group of experiments involved a study of the *in vitro* transfer of acetate to histones by rat liver DNP in the presence of various concentrations of hydrocortisone. Preliminary experiments gave indications that a stimulation of histone acetylation may occur under these conditions, however, upon closer investigation, it was concluded that this is not the case.

Unless otherwise stated the incubation medium for these

experiments contained 0.19 M sucrose, 20 mM glucose, 25 mM phosphate buffer, pH 8, 12 mM NaCl, and 0.01 μc acetyl-^{14}C-CoA. Other conditions, including the isolation of histones and determination of specific activities, were as described in Materials and Methods.

Purity of hydrocortisone preparations

In preliminary experiments hydrocortisone preparations were used which were obtained from the hospital pharmacy. Since this material would not dissolve completely in water at the required dilution it was suspected that this preparation contained some contaminants. An interesting report related to this finding was the observation that 21-dehydrocorticosteroids bind irreversibly to arginine-rich histones and that these compounds frequently contaminate steriod preparations, leading to the suggestion that corticosteroid alcohols react with histones.[170] Other findings have shown that 21-dehydrocortisol was much more effective than hydrocortisone in the *in vitro* stimulation of RNA synthesis in rat liver nuclei.[171] This may explain the preliminary findings in which there appeared to be a positive effect on the acetylation of histones *in vitro*. Subsequent experiments reported here were carried out with hydrocortisone from Sigma Chemical Company.

Use of more samples (Table XI)

In some of the preliminary experiments the difference between samples was not statistically significant although there was a suggestion of an increase with increasing concentration of hydrocortisone from 10^{-10}-10^{-7} M. When 5 samples were run for each condition the data summarized in Table XI were obtained. As can be seen no significant differences could be observed between controls and experimentals.

Use of adrenalectomized rat (Table XII)

Because the adrenal gland is responsible for hydrocortisone production, there is a possibility that removal of the adrenal gland might lower the levels of circulating hydrocortisone and that under these conditions perhaps an *in vitro* effect of the hormone on acetyltransferase activity may be observed. A male rat kept on a salt water diet for 72 hours after adrenalectomy was sacrificed along with a control. The DNP was isolated and incubated as described in Materials and Methods. The results in Table XII were obtained. From this experiment it was concluded that there is little or no affect of hydrocortisone on acetyltransferase activity under these conditions.

Table XI

Effect of Increasing Concentrations of Hydrocortisone on *In Vitro* Acetylation of Histones in DNP

Conc. Hydrocortisone in Incubation Media	Specific Activity (cpm/mg)
0	6040 ± 388
10^{-10}M	6461 ± 407
10^{-9}M	6530 ± 361
10^{-7}M	5969 ± 644

The data presented are the results of 1 experiment and are expressed as specific activity (counts per minute per mg) of histones plus or minus the standard error.

Table XII

Effect of Hydrocortisone on *In Vitro* Acetylation of Histones in Liver DNP from an Adrenalectomized Rat

Conc. Hydrocortisone in Incubation Media	Specific Activity (cpm/mg)	
	Adrenalectomized Rat	Control Rat
0	5323 ± 1050	6821 ± 423
10^{-7}M	6371 ± 1050	7247 ± 279

The data presented are the results of 1 experiment using 5 samples for each condition. They are expressed as specific activity (counts per minute per mg) of histones plus or minus the standard error.

Preincubation of DNP with hydrocortisone (Table XIII)

Because Lukas and Sekeris have demonstrated an increase in mammalian RNA polymerase activity *in vitro* upon preincubation of rat liver nuclei with hydrocortisone,[172] it seemed worthwhile to try if an increase in acetyltransferase activity might be observed upon preincubation of DNP with hydrocortisone. In this experiment DNP was preincubated with 10^{-7}M hydrocortisone in the usual incubation medium at 4°C and 37°C for 10 minutes prior to addition of acetyl-^{14}C-CoA. The results in Table XIII were obtained. No significant differences could be observed by the different treatments.

Media changes designed to increase the solubility of DNP during the incubation (Table XIV)

Because DNP is in a suspended particulate state in the salt conditions of the usual incubation medium, it seemed possible that this could be the reason for its unresponsiveness to hydrocortisone, which might otherwise have an effect on its acetylating ability. Therefore, DNP was washed in water, which causes it to swell and become gelatinous, and incubated either in 12 mM NaCl, 25 mM phosphate buffer, pH 8, or water in the presence of acetyl-^{14}C-CoA. From the results shown in Table XIV it was concluded that changing the colloidal state of DNP in this manner does not make it any more responsive to the presence of hydrocortisone, and incubating DNP in the presence of water only decreases acetyltransferase activity.

Table XIII

Effect of Preincubation of DNP with Hydrocortisone on *In Vitro* Acetylation of Histones

Conc. of Hydrocortisone	Specific Activity (cpm/mg)	Conditions of Preincubation
0	4848 ± 354	0 time
10^{-7}M	4280 ± 816	0 time
0	4713 ± 178	10 min. at 4°C
10^{-7}M	4254 ± 499	10 min. at 4°C
0	4011 ± 319	10 min. at 37°C
10^{-7}M	3163 ± 785	10 min. at 37°C

After preincubation, the acetyl-^{14}C-CoA was added to the samples and the same medium was used for the incubation. The data are the results of 1 experiment in which 4 samples were used for each condition. They are expressed as specific activity of histones (counts per minute per mg) plus or minus standard error.

Table XIV

Effect of Increasing the Solubility of DNP on *In Vitro* Acetylation of Histones in the Presence of Hydrocortisone

Pre-treatment of DNP	Incubation Media	Specific Activity (cpm/mg)
Usual	Usual	9891 ± 117
Usual	H_2O	2931 ± 128
H_2O	Usual	12006 ± 365
H_2O	H_2O	346 ± 138
Usual	H_2O + 10^{-7}M hydrocortisone	2467 ± 23
H_2O	Usual +10^{-7}M hydrocortisone	9259 ± 521
H_2O	H_2O + 10^{-7}M hydrocortisone	201 ± 87

The results are expressed as specific activity (counts per minute per mg) of histones plus or minus the standard error.

Effect of in vivo administration of hydrocortisone on the in vitro acetylation of histones (Table XV)

Another approach to this problem was a study of the effect of the in vivo injection of hydrocortisone on the in vitro acetylation of histones. By measuring the in vivo uptake of ^{3}H-acetate into histones others (see Introduction) have concluded that the in vivo injection of hydrocortisone increased the acetylation of histones. Following similar conditions (time course, dose, etc.) we found no stimulation of the in vitro acetylation of histones by DNP following the injection of hydrocortisone.

In this experiment a 450 gm male rat was injected with 10 mg of hydrocortisone subcutaneously between the shoulder and sacrificed 1½ hours later. DNP was isolated and incubated as usual. The results are summarized in Table XV.

Effect of phenobarbital and liver regeneration on the in vitro acetylation of histones in DNP (Table XVI)

Injection of phenobarbital or removal of part of the liver are known means of stimulating RNA synthesis in liver cells. In the case of liver regeneration an increase in the in vivo acetylation of histones can be observed. The effect of these treatments on the in vitro acetylation of histones in DNP was therefore studied. The median and left lateral lobe of the liver of one 450 gm rat was surgically extirpated 3½ hours before sacrifice. DNP was isolated from the

Table XV

Effect of *In Vivo* Administration of Hydrocortisone on *In Vitro* Acetylation of Histones in DNP

Treatment	Specific Activity (cpm/mg)
Control	7858 ± 688
Hydrocortisone	8220 ± 565

These data represent the results of 1 experiment utilizing 7 samples from each rat. They are expressed as specific activity (counts per minute per mg) of histones plus or minus the standard error.

Table XVI

Effect of Phenobarbital and Liver Regeneration on *In Vitro* Acetylation of Histones in DNP

Treatment	Specific Activity (cpm/mg)
Control	7858 ± 688
Phenobarbital	8559 ± 518
Regenerating Liver	6899 ± 605

These data represent the results of 1 experiment utilizing 7 samples from each rat. They are expressed as specific activity (counts per minute per mg) of histones plus or minus the standard error.

remaining regenerating portion and incubated as usual. One 390 gm rat was injected with phenobarbital (75 mg/kg) 21 hours before sacrifice. DNP was isolated from the liver and incubated as usual. Results are shown in Table XVI. As can be seen, no significant differences were observed between the control and the treated rats.

These experiments demonstrate the need for caution when drawing conclusions concerning cause and effect relationships from *in vivo* observations. Under these conditions no stimulation of acetylating activity of DNP by hydrocortisone can be demonstrated. Therefore, it is likely that increased acetate incorporation into histones observed *in vivo* is an indirect effect of the hormone, and is not due to a modification in the amount or activity of acetyltransferase. The same conclusion appears to be true for the experiment in which the effects of phenobarbital and liver regeneration were studied.

LIST OF REFERENCES

1. V. R. Potter, R. A. Gerbert, and H. C. Pitot, in G. Weber, Advances in Enzyme Regulation, Vol. IV, Oxford Press, New York, 1966, p. 247.

2. F. Jacob and J. Monod, J. Mol. Biol., 3 (1961) 318.

3. W. Gilbert and B. Muller-Hill, Proc. Natl. Acad. Sci. U. S., 56 (1966) 1891.

4. M. Ptashne, Proc. Natl. Acad. Sci. U. S., 57 (1967) 306.

5. W. Gilbert and B. Muller-Hill, Proc. Natl. Acad. Sci. U. S., 58 (1967) 2415.

6. M. Ptashne, Nature, Lond., 214 (1967) 232.

7. A. E. Mirsky and H. Ris, Nature, Lond., 163 (1949) 666.

8. R. Vendrely and C. Vendrely, Experientia, 4 (1948) 434.

9. B. J. McCarthy and B. H. Hoyer, Proc. Natl. Acad. Sci. U. S., 52 (1964) 915.

10. B. H. Hoyer, B. J. McCarthy, and E. T. Bolton, Sci., 144 (1964) 959.

11. J. B. Gurdon, J. Embryol. Exp. Morphol., 10 (1962) 622.

12. R. Briggs and T. J. King, J. Morphol., 100 (1957) 269.

13. A. L. Dounce, G. H. Tishoff, S. R. Barnett, and R. M. Freer, J. Gen. Physiol., 33 (1950) 629.

14. E. Heitz, Jahrb. Wiss. Botan., 69 (1928) 762.

15. P. F. Davidson and J. A. V. Butler, Biochim. Biophys. Acta, 49 (1956) 286.

16. C. F. Crampton, J. Biol. Chem., 227 (1957) 495.

17. A. E. Mirsky and A. Pollister, J. Gen. Physiol., 30 (1946) 117.

18. H. Busch, W. J. Steele, L. Hnilica, C. Taylor, and H. Mavioglu, J. Cell. Comp. Physiol., 62, Suppl. (1963) 95.

19. W. J. Steele and H. Busch, Cancer Res., 23 (1963) 1153.

20. S. B. Weiss, Proc. Natl. Acad. Sci. U. S., 46 (1960) 1020.

21. H. Busch, Histones and Other Nuclear Proteins, Academic Press, New York, 1965.

22. R. C. Huang and J. Bonner, Proc. Natl. Acad. Sci. U. S., 54 (1965) 960.

23. R. C. Huang, Fed. Proc., 26 (1967) 603.

24. H. Ris, Colloq. Ges. Physiol. Chem., 9 (1959) 1.

25. H. Ris and B. L. Chandler, Cold Spring Harbor Symp. Quant. Biol., 28 (1963) 1.

26. E. J. DuPraw, Proc. Natl. Acad. Sci. U. S., 53 (1965) 161.

27. E. J. DuPraw, Develop. Biol., 12 (1965) 53.

28. E. J. DuPraw and G. F. Bahr, J. Cell Biol., 38 (1968) 38A.

29. G. P. Georgiev, Regulatory Mechanisms for Protein Synthesis in Mammalian Cells in A. San Pietro, M. Lamborg, and F. Kenny, Academic Press, New York, 1968, p. 25.

30. V. C. Littau, V. G. Allfrey, J. H. Frenster, and A. E. Mirsky, Proc. Natl. Acad. Sci. U. S., 52 (1964) 93.

31. J. H. Frenster, V. G. Allfrey, and A. E. Mirsky, Proc. Natl. Acad. Sci. U. S., 50 (1963) 1026.

32. R. J. Britten and D. E. Kohne, Sci., 161 (1968) 529.

33. D. D. Brown and I. B. Dawid, Sci., 160 (1968) 272.

34. O. L. Miller, Jr. and B. R. Beatty, Sci., 164 (1969) 955.

35. R. R. Burgess, A. A. Travers, J. J. Dunn, and E. K. F. Bautz, Nature, Lond., 221 (1969) 43.

36. L. R. Thompson and B. J. McCarthy, Biochem. Biophys. Res. Comm., 30 (1968) 166.

37. H. Stein and P. Hausen, Eur. J. Biochem., 14 (1970) 270.

38. E. Stedman and E. Stedman, Phil. Trans. Roy. Soc. London, B235 (1951) 565.

39. R. C. Huang and J. Bonner, Proc. Natl. Acad. Sci. U. S., 48 (1962) 1216.

40. B. Sonnenberg and G. Zubay, Proc. Natl. Acad. Sci. U. S., 54 (1965) 415.

41. J. Bonner and R. C. Huang, Biochem. Biophys. Res. Comm., 22 (1966) 211.

42. J. A. V. Butler and A. R. Chipperfield, Nature, Lond., 215 (1967) 1188.

43. R. R. Clark and P. Byvoet, In Press.

44. K. Marushige and J. Bonner, J. Mol. Biol., 15 (1966) 160.

45. L. Berlowitz, D. J. Pallotta, and C. H. Sibley, J. Cell Biol., 39 (1968) 13a.

46. J. H. Frenster, Nature, Lond., 206 (1965) 1269.

47. H. Swift, in J. Bonner and P. Ts'o, Nucleohistones, Holden-Day, San Francisco, 1964, p. 169.

48. W. Beerman, Amer. Zoologist, 3 (1963) 23.

49. A. E. Mirsky and H. Ris, J. Gen. Physiol., 34 (1951) 475.

50. M. M. Daly, V. G. Allfrey, and A. E. Mirsky, J. Gen. Physiol., 36 (1952) 173.

51. V. G. Allfrey, M. M. Daly, and A. E. Mirsky, J. Gen. Physiol., 38 (1955) 415.

52. V. G. Allfrey., A. E. Mirsky, and S. Osawa, J. Gen. Physiol., 40 (1957) 451.

53. H. G. Rose and J. H. Frenster, Biochim. Biophys. Acta, 106 (1966) 577.

54. R. M. Johnson and S. J. Albert, J. Biol. Chem., 200 (1953) 335.

55. S. Sperti, M. Lorini, L. A. Pinna, and V. Moret, Biochim. Biophys. Acta, 82 (1964) 476.

56. T. C. Spelsberg and L. S. Hnilica, Biochim. Biophys. Acta, 195 (1969) 63.

57. J. Paul and R. S. Gilmour, J. Mol. Biol., 34 (1968) 305.

58. I. Bekhor, G. M. Kung, and J. Bonner, J. Mol. Biol., 39 (1969) 351.

59. K. Marushige, D. Brutlag, and J. Bonner, Biochemistry, 7 (1968) 3149.

60. T. C. Spelsberg and L. S. Hnilica, Federation Proc., 27 (1968) 336.

61. T. C. Spelsberg, S. Tankersley, and L. S. Hnilica, Proc. Natl. Acad. Sci. U. S., 62 (1969) 1218.

62. T. C. Spelsberg, S. Tankersley, and L. S. Hnilica, Experientia, 25 (1969) 129.

63. P. Chambon, M. Ramuz, P. Mandel, and J. Doly, Biochim. Biophys. Acta, 157 (1968) 504.

64. P. Chambon, H. Karon, M. Ramuz, and P. Mandel, Biochim. Biophys. Acta, 157 (1968) 520.

65. T. C. Spelsberg and L. S. Hnilica, Biochim. Biophys. Acta, 195 (1969) 55.

66. E. W. Johns and J. A. V. Butler, Biochem. J., 82 (1962) 15.

67. E. W. Johns, Biochem. J., 92 (1964) 55.

68. D. M. P. Phillips and E. W. Johns, Biochem. J., 94 (1965) 127.

69. E. W. Johns, Biochem. J., 105 (1967) 611.

70. P. Sautiere, W. C. Starbuck, C. Roth, and H. Busch, J. Biol. Chem., 243 (1968) 5899.

71. G. Quagliarotti, Y. Ogawa, C. W. Taylor, P. Sautiere, J. Jordan, W. C. Starbuck, and H. Busch, J. Biol. Chem., 224 (1969) 1796.

72. Y. Ogawa, G. Quagliarotti, J. Jordan, C. W. Taylor, W. C. Starbuck, and H. Busch, J. Biol. Chem., 244 (1969) 4387.

73. R. J. DeLange, D. M. Fambrough, E. L. Smith, and J. Bonner, J. Biol. Chem., 243 (1968) 5906.

74. R. J. DeLange, D. M. Fambrough, E. L. Smith, and J. Bonner, J. Biol. Chem., 244 (1969) 319.

75. R. J. DeLange, D. M. Fambrough, E. L. Smith, and J. Bonner, J. Biol. Chem., 244 (1969) 5669.

76. R. H. Stellwagen and R. D. Cole, J. Biol. Chem., 243 (1968) 4456.

77. J. M. Kinkade, J. Biol. Chem. Federation Proc., 27 (1968) 773.

78. M. Bustin and R. D. Cole, Arch. Biochem. Biophys., 127 (1968) 457.

79. M. Bustin and R. D. Cole, J. Biol. Chem., 243 (1968) 4500.

80. J. Bonner and R. C. Huang, in A. V. S. deReuck and J. Knight, Histones: Their Role in the Transfer of Genetic Information, Ciba Found. Study Group 24, J. & A. Churchill, Ltd., London, 1966, p. 18.

81. P. J. Byvoet, J. Mol. Biol., 17 (1966) 311.

82. R. S. Piha, M. Cuenod, and H. Waelsch, J. Biol. Chem., 241 (1966) 2397.

83. L. R. Gurley, J. M. Hardin and W. H. Langham, Federation Proc., 27 (1968) 797.

84. E. Robbins and T. W. Borun, Proc. Natl. Acad. Sci. U. S., 57 (1967) 409.

85. D. M. Prescott, J. Cell Biol., 31 (1966) 1.

86. J. A. Hardin, G. E. Einem, and D. T. Lindsay, J. Cell Biol., 32 (1967) 709.

87. L. R. Gurley and J. M. Hardin, Arch. Biochem. Biophys., 128 (1968) 285.

88. J. Spalding, K. Kajiwara, and G. C. Mueller, Proc. Natl. Acad. Sci. U. S., 56 (1966) 1535.

89. V. G. Allfrey, B. G. T. Pogo, A. O. Pogo, L. J. Kleinsmith, and A. E. Mirsky, in A. V. S. deReuck and J. Knight, Histones: Their Role in the Transfer of Genetic Information, Ciba Found. Study Group 24, J. & A. Churchill, Ltd., London, 1966, p. 42.

90. C. E. Sekeris, K. E. Sekeris, and D. Z. Gallwitz, Physiol. Chem., 348 (1967) 1660.

91. D. G. Comb, N. Sarkar, and C. J. Pinzino, J. Biol. Chem., 241 (1966) 1857.

92. E. L. Gershey, G. W. Haslett, G. Vidali, and V. G. Allfrey, J. Biol. Chem., 244 (1969) 4871.

93. E. L. Gershey, G. Vidali, and V. G. Allfrey, J. Biol. Chem., 243 (1968) 5018.

94. G. Vidali, E. L. Gershey, and V. G. Allfrey, J. Biol. Chem., 243 (1968) 6361.

95. T. A. Langan and L. Smith, Federation Proc., 26 (1967) 603.

96. V. G. Allfrey, R. Faulkner, and A. E. Mirsky., Proc. Natl. Acad. Sci. U. S., 51 (1964) 786.

97. S. Kim and W. K. Paik, J. Biol. Chem., 240 (1965) 4629.

98. B. D. Patterson and D. D. Davies, Biochem. Biophys., Res. Comm., 34 (1969) 791.

99. T. Tidwell, V. G. Allfrey, and A. E. Mirsky, J. Biol. Chem., 243 (1968) 707.

100. M. G. Ord and L. A. Stocken, Biochem. J., 107 (1968) 403.

101. T. A. Langan, Regulatory Mechanisms for Protein Synthesis in Mammalian Cells, in A. San Pietro, M. Lamborg, and F. Kenny, Academic Press, New York, 1968, p. 101.

102. M. H. Meisler and T. A. Langan, J. Cell Biol., 35 (1967) 91a.

103. T. A. Langan, Sci., 162 (1968) 579.

104. T. A. Langan, Proc. Natl. Acad. Sci. U. S., 64 (1969) 1276.

105. L. J. Kleinsmith, V. G. Allfrey, and A. E. Mirsky, Proc. Natl. Acad. Sci. U. S., 55 (1966) 1182.

106. E. L. Gershey and L. J. Kleinsmith, Biochim. Biophys. Acta, 194 (1969) 519.

107. J. H. Frenster, Nature, Lond., 206 (1965) 680.

108. W. B. Benjamin and R. M. Goodman, Sci., 166 (1969) 629.

109. V. G. Allfrey, Can. Res., 26 (1966) 2026.

110. B. G. T. Pogo, A. O. Pogo, V. G. Allfrey, and A. E. Mirsky, Proc. Natl. Acad. Sci. U. S., 59 (1968) 1337.

111. B. G. T. Pogo, V. G. Allfrey, and A. E. Mirsky, Proc. Natl. Acad. Sci. U. S., 55 (1966) 805.

112. B. G. T. Pogo, V. G. Allfrey, and A. E. Mirsky, J. Cell Biol., 35 (1967) 477.

113. J. A. Wilhelm and K. S. McCarty, Can. Res., 30 (1970) 418.

114. V. G. Allfrey, Can. Cancer Conf., 6 (1964) 313.

115. D. Gallwitz and C. E. Sekeris, Hoppe-Seyler's Z. Physiol. Chem., 350 (1969) 150.

116. D. Gallwitz, Hoppe-Seyler's Z. Physiol. Chem., 351 (1970) 1050.

117. H. Nohara, T. Takahashi, and K. Ogata, Biochim. Biophys. Acta, 154 (1968) 529.

118. D. Gallwitz, Biochem. Biophys. Res. Comm., 32 (1968) 117.

119. D. Gallwitz, Biochem. Biophys. Res. Comm., 40 (1970) 236.

120. D. M. P. Phillips, Biochem. J., 87 (1963) 258.

121. D. M. P. Phillips, Biochem. J., 107 (1968) 135.

122. A. Inoue and D. Fujimoto, Biochem. Biophys. Res. Comm., 36 (1969) 146.

123. P. R. Libby, Biochim. Biophys. Acta, 213 (1970) 234.

124. V. G. Allfrey and A. E. Mirsky, Cold Spring Harbor Symp., 28 (1963) 247.

125. V. G. Allfrey and A. E. Mirsky, in J. Bonner and P. Ts'o, Nucleohistones, Holden-Day, San Francisco, 1964, p. 267.

126. V. G. Allfrey, Regulatory Mechanisms for Protein Synthesis in Mammalian Cells, in A. San Pietro, M. Lamborg, and F. Kenny, Academic Press, New York, 1968, p. 65.

127. V. G. Allfrey, B. G. T. Pogo, V. C. Littau, E. L. Gershey, and A. E. Mirsky, Sci., 159 (1968) 314.

128. U. Clever and E. G. Ellgaard, Sci., 169 (1970) 373.

129. J. P. P. V. Monjardino and A. J. MacGillivray, Exp. Cell Res., 60 (1970) 1.

130. D. Killander and R. Rigler, Exp. Cell Res., 39 (1965) 710.

131. R. B. Church and B. J. McCarthy, J. Mol. Biol., 23 (1967) 459.

132. A. O. Pogo, V. G. Allfrey, and A. E. Mirsky, Proc. Natl. Acad. Sci. U. S., 56 (1966) 550.

133. K. Tsukada and I. Lieberman, J. Biol. Chem., 240 (1965) 1731.

134. J. Doly, M. Ramuz, P. Mandel and P. Chambon, Biochim. Biophys. Acta, 108 (1965) 521.

135. M. M. Thaler and C. A. Villee, Proc. Natl. Acad. Sci. U. S., 58 (1967) 2055.

136. F. Takaku, K. Nakao, T. Ono, and H. Terayama, Biochim. Biophys. Acta, 195 (1969) 396.

137. M. Feigelson, P. R. Gross, and P. Feigelson, Biochim. Biophys. Acta, 55 (1962) 495.

138. F. T. Kenny and F. J. Kull, Proc. Natl. Acad. Sci. U. S., 50 (1963) 493.

139. D. L. Greenman, W. D. Wicks, and F. T. Kenny, J. Biol. Chem., 240 (1965) 4420.

140. P. R. Libby, Biochem. Biophys. Res. Comm., 31 (1968) 59.

141. G. H. Hogeboom, W. C. Schneider, and M. J. Striebich, J. Biol. Chem., 196 (1952) 111.

142. J. Chauveau, Y. Moule, and C. Rouiller, Exp. Cell Res., 11 (1956) 317.

143. D. M. Berkowiz, T. Kakefuda, and M. B. Sporn, J. Cell Biol., 42 (1969) 851.

144. V. G. Allfrey, V. C. Littau, and A. E. Mirsky, J. Cell Biol., 21 (1964) 213.

145. E. W. Johns, D. M. P. Phillips, P. Simpson, and J. A. V. Butler, Biochem. J., 77 (1960) 631.

146. J. A. V. Butler and P. Cohn, Biochem. J., 87 (1963) 330.

147. O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randall, J. Biol. Chem., 193 (1951) 265.

148. G. Schmidtt and S. J. Thanhauser, J. Biol. Chem., 161 (1945) 83.

149. P. Byvoet, Analyt. Biochem., 13 (1965) 314.

150. K. Burton, Biochem. J., 62 (1956) 315.

151. S. Bondy and S. Roberts, Federation Proc., 29 (1970) 471, No. 2.

152. G. A. Bray, Anal. Biochem., 1 (1960) 279.

153. E. W. Johns and S. Forrester, Eur. J. Biochem., 8 (1969) 547.

154. D. D. Cunningham and D. F. Steiner, Biochim. Biophys. Acta, 145 (1967) 834.

155. S. B. Weiss, Proc. Natl. Acad. Sci. U. S., 46 (1960) 1020.

156. K. J. Narita, J. Am. Chem. Soc., 81 (1959) 1751.

157. E. W. Johns, Biochem. J., 92 (1964) 55.

158. S. Raymond, Clin. Chem., 8 (1962) 455.

159. B. M. Olivera, R. C. Huang, and N. Davidson, Ber.d. Bunsengesell. f. physik. Chem., 68 (1964) 802.

160. G. Zubay and P. Doty, J. Mol. Biol., 1 (1959) 1.

161. H. Lineweaver and D. Burk, J. Am. Chem. Soc., 56 (1934) 658.

162. M. L. Petermann and C. M. Lamb, J. Biol. Chem., 176 (1948) 685.

163. S. L. Commerford, M. J. Hunter, and J. L. Oncley, J. Biol. Chem., 338 (1963) 2123.

164. G. E. Perlmann, J. Biol. Chem., 241 (1966) 153.

165. T. C. Chou and F. Lipmann, J. Biol. Chem., 196 (1952) 89.

166. J. F. Berry and V. P. Whittaker, Biochem. J., 73 (1959) 447.

167. H. Tabor, A. H. Mehler, and E. R. Stadman, J. Biol. Chem., 204 (1953) 127.

168. P. Byvoet, Biochim. Biophys. Acta, 160 (1968) 217.

169. P. R. Libby, Biochim. Biophys. Acta, 213 (1970) 234.

170. C. Monder and M. C. Walker, Federation Proc., 29 (1970) 908, No. 2.

171. E. Ohtsuka and S. S. Koide, Biochem. Biophys. Res. Comm., 35 (1969) 648.

172. I. Lukas and C. E. Sekeris, Biochim. Biophys. Acta, 134 (1967) 85.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Professor of Zoology

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This dissertation was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1970

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